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A better understanding of the progression of a primary breast tumor to its metastatic state is crucial to develop more direct forms of screenings and therapy. Members of the Rho GTPases, in particular Rac1, play important roles in cellular processes implicated in tumor progression, such as proliferation, adhesion and invasion. To identify target genes of Rac1 which mediate its effects on the above processes, we applied cDNA-RDA and microarray analyses. These experiments resulted in the identification of 85 independent gene fragments (among them 23 novel genes) which showed altered expression levels as a result of Rac1V12 and Rac1N17 expression. The difference in mRNA abundance of twenty genes has been reconfirmed by northern blot analysis. Among them are previously identified genes associated with tumorigenesis and/or invasion, such as cyclin D1, COX-2, CDO, ICAP1 and NF-kappa B. We focussed our efforts on the characterization of cyclin D1, COX-2 and more recently ICAP1 with respect to mediating Rac1's effect on cell proliferation and invasion. We obtained evidence supporting a role for COX-2 in Rac1V12-triggered increase in cell growth. The further characterization of the other cDNAs is likely to identify additional relevant Rac1 targets and is presently ongoing.

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Table of Contents

Cover	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body	4
Key Research Accomplishments.....	9
Reportable Outcomes	9
Conclusions.....	11
References.....	12
Appendices.....	15

INTRODUCTION

The major goal of this proposal is to obtain a better understanding of the molecular mechanisms by which a primary tumor progresses to its metastatic state. The progression of a breast tumor cell to its metastatic state is generally the major cause of morbidity and mortality of breast cancer patients. The process of primary tumor progression to a metastatic stage is complex and consists of multiple steps involving aberrant functions of the tumor cell, including increased local proteolysis, degradation of extracellular matrix components, invasion, adhesion to and migration through the vascular basement membrane, and proliferation at distant sites (Kleiner and Stetler-Stevenson, 1999; Liotta et al., 1991; Mignatti et al., 1986; Moscatelli and Rifkin, 1988; Stetler-Stevenson et al., 1992; Stetler-Stevenson and Yu, 2001; Welch et al., 2000). Tumor progression is believed to be the result of multiple alterations, including genetic alterations and changes in gene expression, resulting in loss of normal cellular regulation (Nicolson, 1991; Nicolson, 1998). Over the past few years, members of the Rho GTPases, in particular Rac1, have emerged as key players in adhesion, invasiveness, proliferation and metastasis (reviewed in Boettner and Van Aelst, 2002; Schmitz et al, 2000; see attachments). We postulated that the identification and characterization of the target molecules involved in Rac1-GTPase-specific effects on invasion and metastasis will provide more insights into the mechanism of tumor progression, as well as possibly identify additional diagnostic markers and new targets for therapy. We have utilized cDNA-RDA (representational difference analysis of cDNA) in combination with microarray technique to identify genes which are up- or downregulated as a result of Rac1 expression. As summarized below, we succeeded in the isolation of several interesting potential genes, whose expression levels are altered as a result of constitutively active and dominant negative Rac1 mutant expression in the breast epithelial cell line, Mcf7. Furthermore, we obtained evidence for a role of some of these genes in mediating Rac1's effects on cell proliferation and invasion.

BODY

To identify target genes of Rac1 which mediate its effects on invasion and metastasis, we proposed the following specific aims: 1) Identify genes that are differentially expressed in breast epithelial cells which do and do not express constitutively active and dominant negative Rac1 mutants; 2) Analyze and initially characterize the isolated candidate genes; and 3) Determine a role for the candidate gene products in invasion and metastasis. An overview of the different steps we took to accomplish these aims as well as our achievements are outlined below.

1. Identify genes that are differentially expressed in breast epithelial cells, which do and do not express Rac1 mutants.

We opted to perform cDNA-RDA to identify genes that are differentially expressed in breast epithelial cells which do and do not express constitutively active and dominant negative Rac1 mutants (see below, 1b). In order for cDNA-RDA (or any method developed to identify genes that are expressed at different levels) to be successful, the choice and preparation of the biological sample is important. One has to be able to discern effects that arise as a result of the experimental change from effects that are artificial. Towards this end, we opted to make use of an inducible expression system to express the Rac1 mutants. In this case, the samples for comparisons are paired as ideally as possible. They are derived from the same population, and the only perturbations are the presence of inducer and the expression of Rac1 mutants.

a) Generation of epithelial cell lines inducibly expressing Rac1 mutants.

In our original proposal, we had proposed to establish a breast epithelium T47D cell line expressing Rac1V12 and Rac1N17 under the control of the inducible ecdysone system. However, after extensive analysis, we experienced that this cell line, using the ecdysone system, does not allow for tight expression of the Rac1 mutants, given expression could be observed in the absence of the inducer, ponasterone A. Hence, we decided to make use of a different breast epithelial cell line, namely Mcf7, and the Retroviral Tet-Off system (Clontech) instead of the ecdysone system. As shown for T47D cells, the introduction of Rac1V12 also confers an invasive potential to Mcf7 cells as well as to MDCK cells, when plated on collagen (Sander et al., 1998). We opted for the tetracycline system since Nelson and colleagues observed very tight expression of Rac1V12 and Rac1N17 under the control of the tetracycline-off (Tet-off) system in MDCK cells (Jou and Nelson, 1998). This system is based on an artificial promoter consisting of a tetracycline-response element (TRE) coupled to a CMV minimal promoter, which drives expression of a gene of interest in a tetracycline-dependent manner. The Tet-off system is designed such that medium containing doxycycline activates the tetracycline receptor which secondarily represses the Tet-dependent promoter. Conversely, depletion of doxycycline from the medium causes a conformational change in the tetracycline receptor, resulting in its inactivation and derepression of the target promoter.

We purchased an Mcf7 Tet-off cell line from Clontech, which is ready to use as host. This cell line contains the pTet-off regulatory plasmid that expresses the tetracycline-controlled activator (tTA), a chimeric protein composed of the Tet repressor protein fused to the VP16 activator domain. We then infected the Mcf7 Tet-off cells with retroviruses containing the Rac1 mutants cloned in the pTRE2 response plasmid. The viruses were produced using LinX cells (an amphotropic packaging line provided by Greg Hannon, CSHL). A detailed description of retroviral transduction can be found in our recent chapter in *Methods in Enzymology* (Boettner et al., 2001, see appendix). To evaluate induction of Rac1 expression, we performed Western blot analysis under inducible conditions. We succeeded in establishing Mcf7 clones expressing Rac1V12 and Rac1N17 under the control of the Tet-off system, and found that 48h in the absence of doxycycline was sufficient to induce expression of Rac1V12 and Rac1N17 to a level equal to endogenous Rac1 (data not shown). No expression of the Rac1 mutants was observed in the presence of doxycycline.

b) cDNA-RDA on Rac1V12 and Rac1N17 expressing cell lines.

To identify specific genes that are regulated by Rac1, we applied cDNA-RDA (representational difference analysis of cDNAs). In this technique one cDNA population (called the driver) is hybridized in excess against a second population (the tester) to remove common hybridizing sequences, thereby enriching target sequences unique to the tester (Hubank and Schatz, 1994; Lisitsyn and Wigler, 1993). We performed two sets of cDNA-RDA. In the first experiment, we set out to identify genes that are upregulated by Rac1V12 and Rac1N17 in breast epithelium Mcf7 cells by using cDNAs isolated from cells induced to express Rac1V12 and Rac1N17 as the tester population, and cDNAs isolated from uninduced cells as the driver population. In the second experiment, we used cDNAs isolated from Mcf7 cells induced for Rac1V12 and Rac1N17 expression as the driver, and cDNAs from uninduced cells as the tester, to identify genes which are downregulated by Rac1V12 and Rac1N17 respectively.

An overview of the steps we took is outlined below and summarized in Fig. 1 (see appendix). A detailed protocol to perform cDNA-RDA can be found in our chapter in *Methods*

in Molecular Biology (Schmitz et al., 2002, see appendix). Approximately 5×10^7 cells of each of the two clones (cells containing Rac1V12 or Rac1N17) were grown in the presence of 20 ng/ml doxycycline and each condition was split into two halves. One half of each population was then induced by removal of the doxycycline. mRNA was isolated from cells induced and not induced for Rac1V12 or Rac1N17 expression and the isolated mRNAs were subsequently converted into cDNA. After cDNA synthesis, the tester and driver cDNAs were digested with *DpnII*, adaptor-ligated and amplified by PCR using adaptor primers to generate representations (amplicons). Representations from both tester and driver were cleaved with *DpnII* to remove their adaptors, and then tester amplicon only was ligated to a new oligonucleotide adaptor. Subsequently, the tester amplicon was mixed with excess driver amplicon, the two melted and allowed to reanneal during a hybridization step at 67...C for 20hr. Only the target sequences can be exponentially amplified during a subsequent PCR step with tester-adaptor specific primers (Fig. 1B). These amplified sequences are called difference products1 (DP1). The iterative hybridization-amplification step was repeated once, at higher stringency, and DP1 was used this time as tester with the same driver as above. The resulting difference products are denoted DP2 (see Fig. 1A). To avoid isolating Rac1V12 or Rac1N17 as major DP, when using cDNAs from cells expressing Rac1V12 or Rac1N17 as tester, we added Rac1WT cDNA to supplement the drivers in both cases. The resulting DP2 products were digested with *DpnII* and run on a 2% agarose gel (see Fig. 1C). The whole lane from 800-100bp was cut out, gel purified, subcloned into a bacterial expression vector, and PCR-amplified. Approximately 400 clones from each library (in total 4 libraries from the 4 cDNA-RDA experiments) were selected at random and their individual inserts were PCR-amplified. The PCR amplified inserts were subjected to microarray analysis (see 2, below).

2. Analyze and initially characterize the isolated candidate genes

To confirm the differential expression of the isolated difference products (DPs) obtained from the cDNA-RDA experiments, we made use of micro-array technology (in collaboration with R. Lucito at CSHL). PCR amplified inserts from the isolated DPs were printed onto a glass slide, and then driver and tester cDNAs were differentially labeled with the nucleotide derivatized fluorophores Cy3 and Cy5 during preparation of first strand synthesis with reverse transcriptase (see Fig. 2 in the appendix as an example). The labeled cDNAs were hybridized to the microarray chip and the fluorescence signals resulting from the sequence specific hybridization were quantitated separately for each channel and compared to yield a ratio (Lucito et al., 2000; Schena et al., 1995). This, in other words, generated a measure of the abundance of one message as compared to the other. The clones found to be differentially expressed based on microarraying (fluorescence ratio greater than 2) were subjected to sequencing. A more detailed description can also be found in our chapter in Methods in Molecular Biology (Schmitz et al., 2002, see appendix).

We obtained 85 independent gene fragments, of which 23 were novel, and a total of 37 apparently upregulated and 48 downregulated candidate genes as a result of Rac1V12 or Rac1N17 expression. The obtained genes could be categorized into the following groups: genes encoding nuclear, ribosomal, mitochondrial, membrane-associated, secreted and cytoskeletal proteins. Such a broad spectrum of genes encoding proteins of diverse classes has also been previously observed for genome wide transcriptome screens, such as that for Ras target genes (Zuber et al., 2000).

To further assure that the differences we observed, when comparing two representations of cDNAs, reflect true differences in mRNA abundance, we performed northern blot analysis.

Thus far, we have examined the expression levels of 20 clones in Mcf7 cell lines expressing Rac1V12, Rac1N17, or empty vector, using [α^{32} P] dCTP-random labeled cDNAs (from the 20 candidate genes) as probes, and reconfirmed their difference in mRNA abundance. The identities of the 20 clones and their ratios obtained from the microarray experiments are shown in Table 1 (see appendix). As examples, northern blot analysis for COX-2 and cyclin D1, whose expression levels are upregulated as a result of Rac1V12 induction, are illustrated in Figure 3A. Noteworthy, similar results were obtained for the protein levels of COX-2 and cyclin D1 by western blot analysis, using extracts of Mcf7 cell lines transiently transfected with Rac1V12 or empty vector, and mouse monoclonal antibodies against COX-2 and cyclin D1 (see Fig. 3B, appendix). We are currently finalizing northern blot analysis for all the other clones, including the ESTs. Once this is accomplished, the information on the identity of all the clones will be made available in a manuscript we are presently preparing (Schmitz et al. in preparation). In the mean time, we have obtained all of the ESTs which matched our obtained difference products which did not appear to encode for known proteins. The further identification of their gene products may provide more insight into the molecular mechanism of tumor progression, and may reveal additional diagnostic markers.

3. Determine a role for the candidate gene products in invasion and metastasis.

Among the genes we identified (see Table 1) two clones, namely COX-2 and cyclin D1, particularly attracted our attention as potentially being important in mediating Rac1's effects on breast tumor progression. Both COX-2 and cyclin D1 were upregulated as a result of Rac1V12 induction (4.8- and 3.6-fold, respectively) and both have previously been implicated in tumorigenesis. Hence, we first focussed our efforts on the further characterization of COX-2 and cyclin D1 in the context of Rac1 function. Before discussing our data, a brief summary of previous findings on COX-2 and cyclin D1 pertinent to this study is given below.

Cyclooxygenase-2 (COX-2) is the inducible isoform of prostaglandin H synthase and has been implicated in a broad range of physiological and pathological processes, including inflammation, maintenance of gastrointestinal integrity, and progression of a variety of cancers (Dubois et al., 1998; Taketo, 1998). In contrast to the isoform COX-1, which is constitutively expressed in nearly all normal tissues, COX-2 expression can be upregulated by various stimuli, including inflammatory signals, cytokines, mitogens, and growth factors (Dubois et al., 1998; Williams and DuBois, 1996). The increase in the rate of COX-2 gene transcription is mediated by several promoter elements that respond to multiple signal transduction pathways. Increased levels of COX-2 have been demonstrated in pancreatic, gastric, esophageal, lung, head and neck, GBMs, and metastatic breast tumors (Castelli et al., 1989; Deininger and Schluesener, 1999; Higashi et al., 2000; Howe et al., 2001; Uefuji et al., 2000; Zimmermann et al., 1999). At present, the most compelling evidence for a role of COX-2 in tumorigenesis comes from the findings that several transgenic and carcinogen-induced colon tumor animals express high levels of COX-2 in intestinal polyps. Furthermore, a lack of COX-2 expression results in decreased neoplastic growth and the number of tumors that develop in APC Δ 176 mutant mice (Oshima et al., 1996). The precise mechanism by which COX-2 contributes to tumorigenesis remains to be determined. However, several lines of evidence suggest that COX-2 promotes cell survival (Chang et al., 2000; Sheng et al., 1998).

Cyclin D1 is a key player in cell cycle regulation in mammalian cells. Its activity is essential for passage through the checkpoint (G1 \rightarrow S) of the cell cycle. Cyclin D1 abundance varies within the cell cycle, peaking in G1, and is largely controlled by the rate of cyclin D1

transcription (reviewed in Pestell et al., 1999). The latter is regulated by multiple transcription factors, including NF κ B, AP-1 and STATs. The abundance of cyclin D1 is rate limiting in G1 progression, at least in part because of its role in the formation of holoenzyme complexes with CDK4 and CDK6, which phosphorylate and inactivate the retinoblastoma tumor suppressor (pRB). This causes release of pRB from the E2F transcription factor, activation of E2F responsive genes, and subsequent entry into S phase (Lukas et al., 1995; Pestell et al., 1999). The finding that cyclin D1 is upregulated in Mcf7-Rac1V12-expressing cells is consistent with our previous observation that Rac1V12 can trigger cyclin D1 induction in fibroblasts (Westwick et al., 1997).

Based on our observation that both COX-2 and cyclin D1 expression levels are upregulated by Rac1V12 in the Mcf7 cell line and on the above information, we postulated that COX-2 and cyclin D1 play an important role in mediating some of Rac1's effects on Mcf7 cell growth and invasion. Before addressing this hypothesis, we measured the growth rates and invasive potential of the Mcf7 cells induced for Rac1V12 expression. As shown in Fig. 4 (appendix), the growth rate of Rac1V12 induced Mcf7 cells was markedly faster than the one of uninduced control cells. Cells containing Rac1V12 were plated in 6-well-dishes at a density of 2.5×10^4 per well in medium containing 0.5 % serum with and without 25 M doxycycline (DOX). Cells were then fixed in 10% formalin at indicated time points throughout one week, and stained with crystal violet. Cell-associated dye was extracted with acidic acid, and the optical density was measured at 590 nm, with each value normalized to day 0 (Lin et al., 1998).

As shown in Fig. 5 (appendix), induction of Rac1V12 expression also increased the invasive potential of these cells on collagen. We made use of a modified Boyden chamber assay to measure the invasive potential of cells (Banyard et al., 2000). Briefly, this assay utilized Transwell chambers (Costar), 6.5 mm in diameter with 8 μ m pore size polycarbonate filters. The membranes were coated with collagen overnight and then placed into 24 well tissue culture plates containing DMEM + 5% serum and 1% BSA. Uninduced cells and cells induced for Rac1V12 expression by withdrawal of DC were serum starved, harvested, resuspended in serum free medium containing 1 % BSA, and added to each Transwell chamber. The cells were then incubated for 16 hours to allow them to invade toward the underside of the membrane. Non-invading cells on the upper surface of the filter were removed with a cotton swab, while cells that had migrated to the underside of the membrane were fixed, stained with Hema 3 (Fisher), and the number of cells per membrane counted under a light microscope.

To investigate the involvement of COX-2 in Rac1-mediated effects on cell growth, we made use of a widely used inhibitor of COX-2, NS-398 (Barnett et al., 1994; Castano et al., 2000). Mcf7-Rac1V12 cells maintained with and without DOX were treated with different concentrations (1, 5, 10, 25 and 50 M) of the NS-398 drug, or were left untreated, and growth rates were measured as described above. We noted that the addition of 12.5 M NS-398 could suppress the increased growth rate triggered by Rac1V12 induction, but only affected the growth rate of Rac1V12 uninduced cells slightly (Fig. 6). These data suggest that COX-2 may play a role in mediating the effects of Rac1 on Mcf7 cell proliferation. We did not notice a significant effect of the drug NS-398 on the invasive potential of Rac1V12 expressing cells (data not shown).

To test whether interfering with cyclin D1 expression can reduce the Rac1V12-triggered increase in cell growth, we will make use of a dominant negative cyclin D1 mutant (T156A). The cyclin D1 (T156A) mutant prevents nuclear import of CDK4 and its phosphorylation by CDK-activating kinases. When expressed in cells, it competes with endogenous cyclin D1 and

hence mobilizes CDK4 into cytoplasmic, catalytically inactive complexes (Diehl and Sherr, 1997). We recently succeeded in the generation of stable Rac1V12 lines expressing the dominant negative cyclin D1 (T156A) mutant and empty vector. We are currently examining these cell lines for altered cellular growth and invasive properties following induction of Rac1V12 expression. In addition to cyclin D1 and COX-2, we recently initiated experiments to assess the importance of ICAP1 towards Rac1 function. We found ICAP1 as a cDNA which was upregulated upon Rac1V12 induction and downregulated upon Rac1N17 induction (see Table 1). ICAP1 is a β 1-integrin binding protein and has been suggested to play a role in adhesion and invasion (Chang et al., 1997; Bouvard et al., 1998; Fournier, 2002). Given the role of Rho GTPases in integrin signaling we postulate that ICAP1 may play a role in Rac1's effects on cell adhesion and invasion.

KEY RESEARCH ACCOMPLISHMENTS

- Establishment of MCF7 inducible cell lines expressing dominant active and dominant negative mutants of Rac1.
- Successful completion of cDNA-RDA experiments to identify genes, which are up- or downregulated as a result of Rac1V12 or Rac1N17 expression.
- Implementation of the microarray technique to further analyze the difference products resulting from cDNA-RDA experiments.
- 300 clones derived from four independent cDNA-RDA experiments were found to be differentially expressed by microarray analysis. These clones were sequenced, resulting in 85 independent gene fragments, of which 23 were novel. We found a total of 37 apparently upregulated and 48 downregulated candidate genes.
- We completed northern blot analyses for twenty of the differentially expressed genes (see Table 1) and were able to reconfirm their altered expression levels. Among these genes, several have been previously associated with tumorigenesis. We obtained all of the ESTs for which we had noted matches with our obtained difference products and are in the process of obtaining full-length genes.
- We demonstrated that the inducible Rac1V12 MCF7 cell line we generated shows an increased growth rate and an increased invasive potential upon induction of Rac1V12 expression. We further initiated experiments addressing the role of COX-2 and cyclin D1, and more recently ICAP1, in mediating Rac1's effects on breast tumor progression. We obtained evidence supporting a role for COX-2 in Rac1V12-triggered increase in growth of the breast cell line, MCF7.

REPORTABLE OUTCOME

Manuscripts:

Schmitz, A., Boettner, B., Govek, E.E., and Van Aelst, L. (2000). Rho-GTPases: Signaling, migration, and invasion. Special issue on cell adhesion. *Exp Cell Res*, 261:1-12.

Boettner, B., Herrmann, C., and Van Aelst, L. (2001). Ras and Rap interaction with the AF-6 effector target. *Methods in Enzymology: Regulators and effectors of small GTPases*. (edited by E.E. Balch, J. Der and A. Hall) 332:151-168.

Schmitz, A., Lucito, R., and Van Aelst, L. (2002). Using cDNA-Representational difference analysis (cDNA-RDA) in combination with microarrays. *Methods in Molecular Biology* 189:25-43.

Boettner, B. and Van Aelst, L. (2002). The role of Rho-GTPase in disease development. *Gene*, 286: 155-174.

Schmitz, A., Lucito, R., and Van Aelst, L. (2002). Identification of novel Rac1 target genes in epithelial cells (manuscript in preparation).

Presentations:

Invited speaker (Arndt Schmitz) Seminar, Biozentrum of the University of Basel, Switzerland (August 15, 1999). Host: Gerhard Schwarz. Title: Identification of genes regulated by the Small GTPase Rac using cDNA-RDA.

Invited speaker (Linda Van Aelst) Gordon Research Conference, Singapore (Sept 5-10, 1999). Title: Function and signal transduction of Ras and Rac targets.

Invited speaker (Linda Van Aelst) Seminar, Van Andel Institute, Grand Rapids, MI (Nov. 30, 1999). Host: George Vande Woude. Title: Ras and Rac targets.

Poster presentation (Arndt Schmitz) Schmitz, A., Lucito, R., and Van Aelst, L. Identification of Rac-regulated genes using cDNA-RDA. Poster at the 51. Mosbacher Kolloquium: GTP-binding Proteins: Central Regulators in Cell Biology, Mosbach, Germany, April 2 - 5, 2000.

Invited speaker (Linda Van Aelst) Seminar, SUNY, Stony Brook (April 26, 2000). Host: Wadie Bahou. Title: Isolation of genes involved in Rac induced invasion and metastasis of breast carcinoma cells.

Invited speaker Seminar, McGill University, Montreal, Canada (April 6, 2001). Host: N. Lamarche.

Invited speaker Seminar, University of Virginia, Charlottesville, USA (April 24, 2001). Host: J. Casanova.

Invited speaker FASEB Research Conference on the Ras Superfamily of Small GTP-binding proteins, Snowmass, Colorado, USA (July 15-20, 2000).

Invited speaker and chair: Tyrosine phosphorylation and cell signaling meeting, Cold Spring Harbor, New York, USA (May 16-20, 2001).

Invited speaker Oncogene meeting: Cancer cell signal transduction. Hood College, Frederick, Maryland, USA (June 20-23, 2001)

Chairperson: American Association for Cancer research. Convention Center, New Orleans, LA, USA (March 24-28, 2001).

Invited speaker (Linda Van Aelst) Gordon Research Conference, Oxford, London, England (August 12-17, 2001). Mechanisms of cell signaling.

Invited speaker Seminar, UW-Madison, Wisconsin, USA (March 7, 2002). Host: Maureen Barr.

Invited speaker Seminar, Albert Einstein College, New York, USA (April 2, 2002). Host: Richard Stanley.

Personnel who received pay from the research effort:

Linda Van Aelst (PI)
Benjamin Boettner and Arndt Schmitz (Post-doctoral fellows)
Yi Qin (Research assistant)

Based on the experience Dr. Arndt Schmitz acquired working on this project, he received recently a position as work leader at Shering AG, Germany.

CONCLUSIONS

Members of the Rho GTPases, in particular Rac1, have been well documented to play important roles in adhesion, invasiveness, proliferation and metastasis. These events are crucial steps in the transition of a primary tumor to its metastatic state. Hence, to obtain a better understanding of the underlying molecular mechanism(s) of tumor progression, we set out to identify and characterize target molecules mediating Rac1-specific effects on proliferation, invasion and metastasis.

To identify target genes of Rac1, we applied cDNA-RDA and microarray analyses, which resulted in the identification of 85 independent gene fragments (among them 23 novel genes) which showed altered expression levels as a result of Rac1V12 or Rac1N17 expression. The obtained genes could be categorized into several different groups: genes encoding nuclear, ribosomal, mitochondrial, membrane associated, cell cycle, secreted and cytoskeletal proteins. To reconfirm the differential expression of the above genes, we performed northern blot analyses. Thus far, we reconfirmed the difference in mRNA abundance of twenty genes (see Table 1), most of which were previously identified. Of particular interest is the observation that several of these genes have been previously demonstrated to play a role in tumorigenesis and/or invasion. We focussed our initial attention on the characterization of cyclin D1 and COX-2, and more recently ICAP1, with respect to mediating Rac1's effects on breast tumor progression, and obtained evidence supporting a role for COX-2 in Rac1V12-triggered increase in cell proliferation. Further experiments addressing the importance of the other Rac1 targets in Rac1-triggered effects on adhesion, invasion, and proliferation are under way.

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APPENDIX

Rac1V12 upregulated genes		Rac1V12 downregulated genes		Rac1N17 upregulated genes		Rac1N17 downregulated genes	
	(ratio)		(ratio)		(ratio)		(ratio)
myosin II	2.8	CDO	(3.7)	protein with homology to HE-4	(2.3)	ICAPI	(2.4)
calcyclin	4.8	VH16	(2.2)	vesicle sorting protein VPS35	(2.8)		
hBMP-4	(2.2)	β -1-4 galactosyltransferase	(4.8)				
STAT5	(2.9)	Mitochondrial cytochrome c oxidase					
ribosomal protein (PO)	(3.2)	(cox III)	(3.1)				
dynein	(2.6)	EST3	(4.2)				
hephaestin	(7.2)						
cyclin D	(3.6)						
Cyclooxygenase-2 (COX-2)	(4.8)						
ICAPI	(2.7)						
NF-KB	(6.2)						
EST1	(3.7)						
EST2	(6.1)						

Table 1: Differential expression of genes in Mcf7 cells, resulting of Rac1V12 or Rac1N17 expression. The isolated DPs obtained from the cDNA-RDA experiments were printed onto a glass slide (chip). Tester and driver cDNAs were differentially labeled and hybridized to the microarray chip. The fluorescence signals resulting from the hybridization were quantitated separately for each channel and compared to yield a ratio.

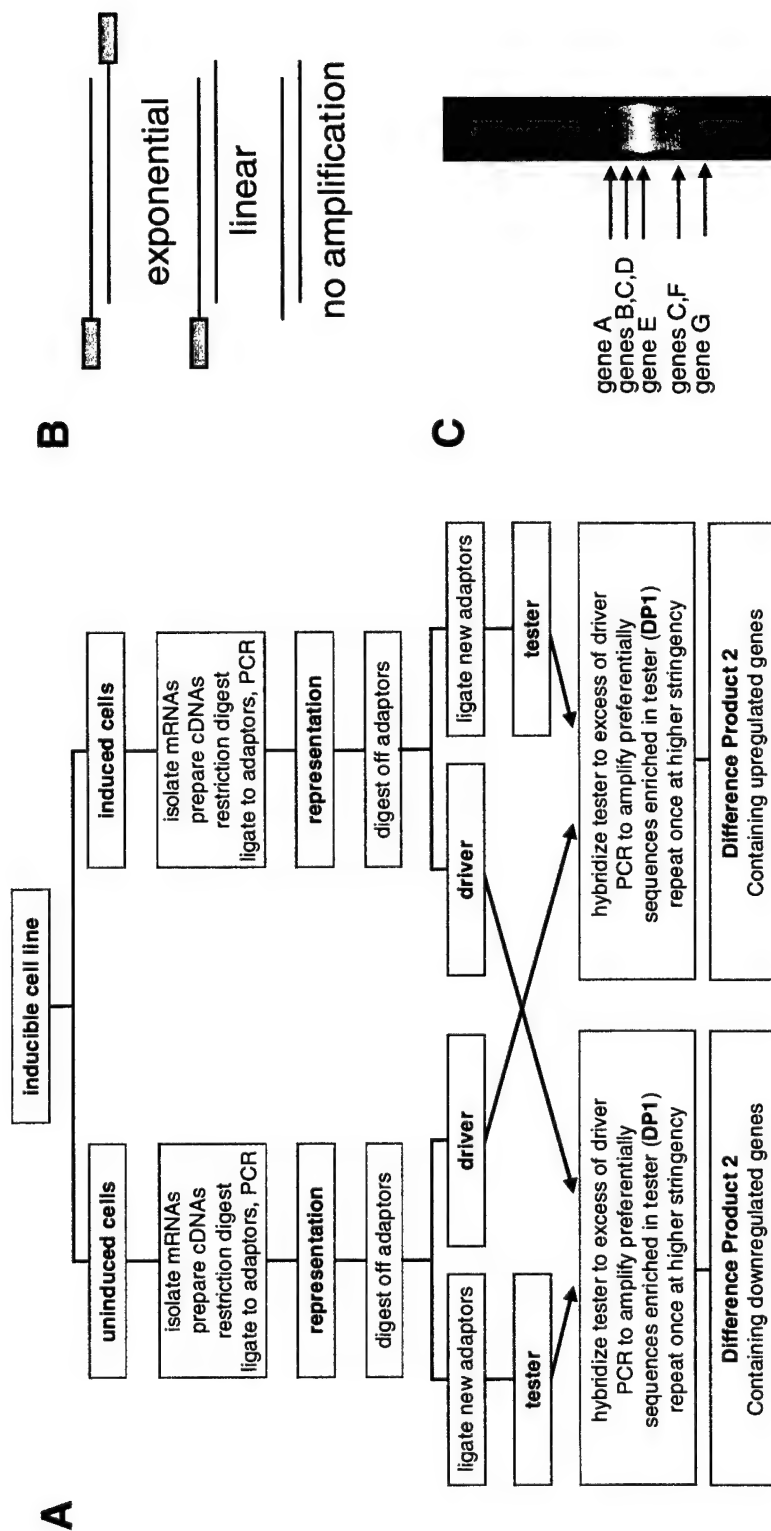


Fig. 1: Principle of cDNA-RDA. A. Flowchart of cDNA-RDA (see text for more detail). B. The three possible outcomes of the tester/driver hybridization. If a sequence is unique to the tester or present at a higher molar ratio in the driver, it will be exponentially amplified. If a sequence is found in both driver and tester to equal amounts, only the strand from the tester population bears the adaptor and the sequence will be linearly amplified. If the sequence is found only in the driver, neither strand contains the adaptor sequence and the sequence will not be amplified. C. A typical difference product after two rounds of cDNA-RDA visualized by agarose gel electrophoresis. A difference product consists of a series of visible bands superimposed on a 'smear'. As indicated, each band may contain fragments of several genes whose different sizes can not be resolved on an agarose gel. Furthermore, candidate genes may be contained in the 'smear'. Finally, different fragments of the same gene can occur at different positions in the gel, since cDNA-RDA is based on digested cDNA.

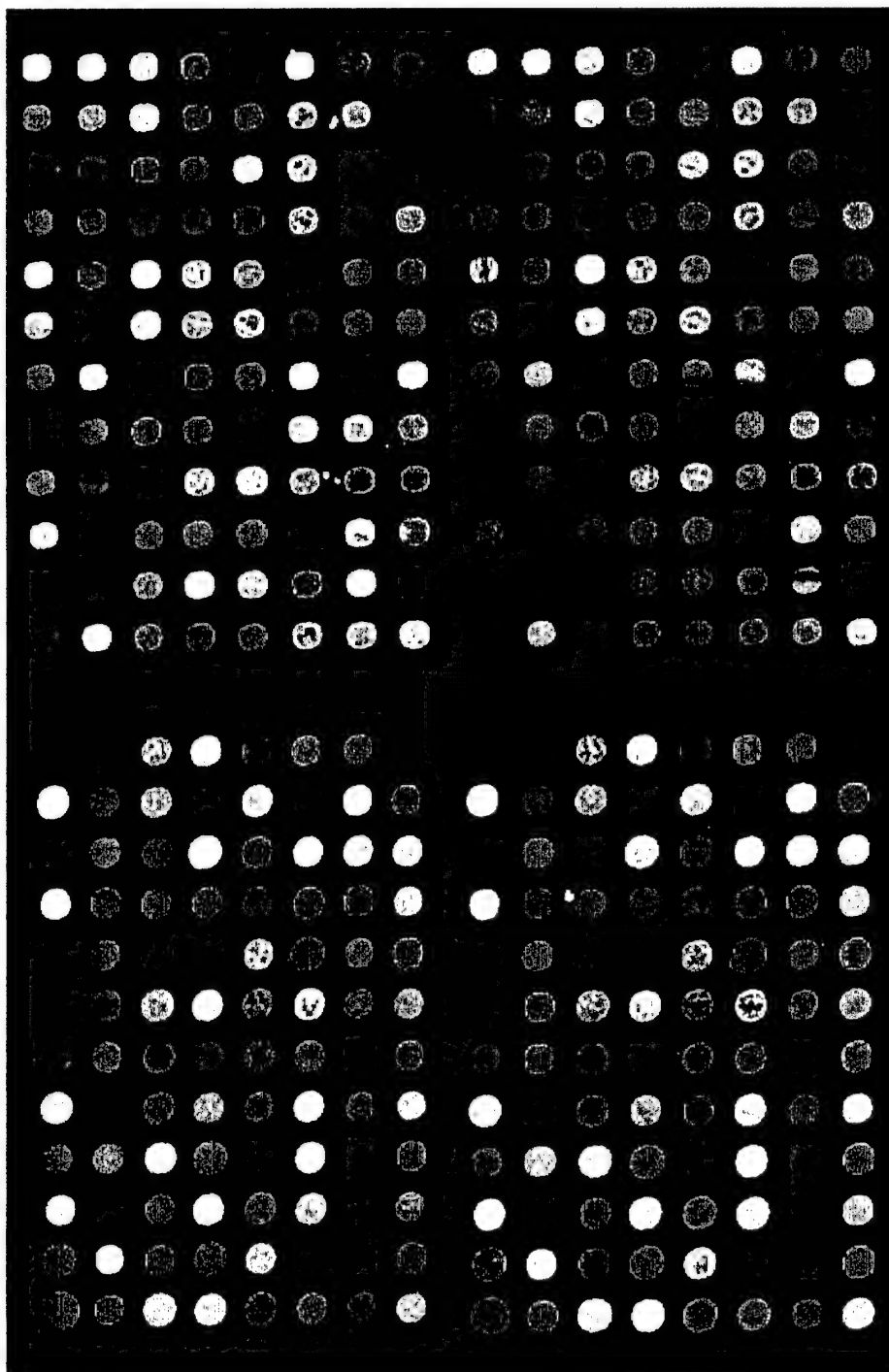


Fig. 2: Analysis of cDNA-RDA products by the microarray technique. Clones derived from a cDNA-RDA experiment using cells induced for Rac1V12 expression as the tester and uninduced cells as the driver were arrayed in duplicate. The array was then hybridized simultaneously to a representation from Rac1V12 induced cells (labeled in green) and to a representation from uninduced cells (labeled in red). The clones with different shades of green represent genes whose expression is upregulated as a result of Rac1V12 expression, whereas yellow clones are transcripts unaffected by Rac1V12 expression. Genes downregulated by Rac1V12 would be identified as red spots, but are absent from the design of this particular experiment. Such, microarraying allows rapid screening of a difference product.

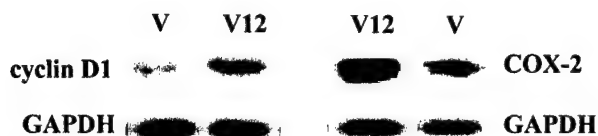


Fig. 3A: Northern blot analysis of cyclin D1 and COX-2 expression. 15 μ g of total RNA were isolated from cells transiently transfected with Rac1V12 or empty vector and assessed by northern blot analysis using cDNA probes for cyclin D1 and COX-2. Load normalization was verified by reprobing blots with a GAPDH cDNA.

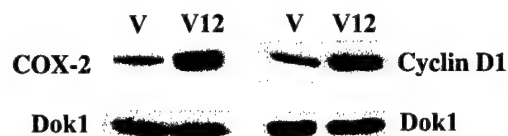


Fig. 3B: Western blot analysis of cyclin D1 and COX-2 expression. Rac1V12- or empty vector-expressing MCF7 cells were lysed and equal protein amounts were subjected to western blot analysis using a monoclonal Ab against COX-2 (Transduction Laboratories) or a monoclonal Ab against cyclin D1 (Pharmingen). Load normalization was verified using a polyclonal Ab against dok1

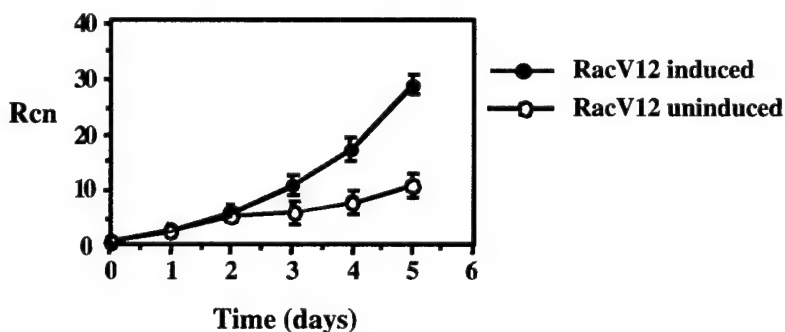


Fig. 4: Growth properties of Rac1V12-expressing MCF7 cells. Growth rates of MCF7 cells induced and uninduced for Rac1V12 expression, determined in the presence of 0.5 % serum. Each value was determined in triplicate and normalized to the cell number at day 0. (Rcn= relative cell number)

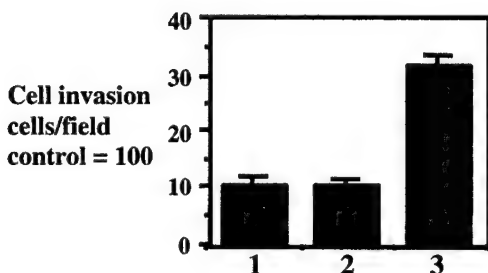


Fig. 5: Cell invasion assay for Rac1V12-expressing MCF7 cells. Cell invasion through collagen was analyzed using a modified Boyden chamber assay (see text). Cells that migrated to the lower side of the membrane were counted from at least 48 different fields (63x magnification) and for three experiments. The averaged values were normalized to those obtained for the parental MCF7 control cells (1= control; 2= Rac1V12 uninduced; 3= Rac1V12 induced).

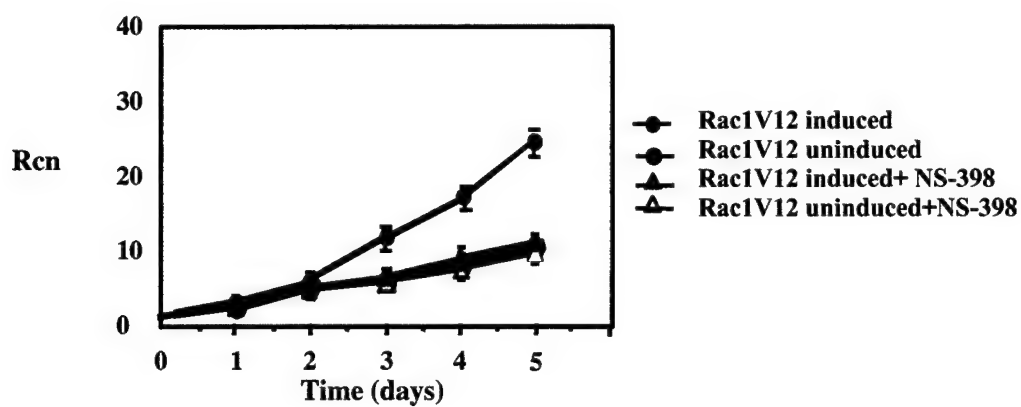


Fig. 6: Effect of COX-2 inhibitor on the growth rate of Rac1V12-expressing Mcf7 cells. Growth rates of Mcf7 cells induced and uninduced for Rac1V12 were treated with 25 M NS-398 or left untreated. Each value was determined in triplicate and normalized to the cell number at day 0. (Rcn = relative cell number)

MINIREVIEW

Rho GTPases: Signaling, Migration, and Invasion

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The acquisition of a motile and invasive phenotype is an important step in the development of tumors and ultimately metastasis. This step requires the abrogation of cell–cell contacts, the remodeling of the extracellular matrix and of cell–matrix interactions, and finally the movement of the cell mediated by the actin cytoskeleton. Evidence for participation of Rho GTPases in migration and invasion is addressed in this review with emphasis on epithelial cells and the contribution of Rho GTPases toward tumor invasion. The Rho GTPases, including Rac, Cdc42, and Rho, have been implicated in the establishment of cell–cell contacts and of cell–matrix interactions crucial to attaining a fully polarized epithelial state, and they are known for their regulation of the actin cytoskeleton and transcriptional activation. Under aberrant conditions, however, they have been implicated in motility, invasion, and some aspects of metastasis. It is well known that Rho GTPases are activated by different classes of transmembrane receptors and that they transmit these signals to their effector proteins. These downstream targets include not only adaptor proteins and kinases which affect the actin cytoskeleton, but also transcription factors leading to expression of genes necessary for the drastic morphological changes which accompany these processes. © 2000 Academic Press

Key Words: Rho GTPases; signaling; motility; actin cytoskeleton; invasion.

INTRODUCTION

The acquisition of migratory and invasive properties are key events in the oncogenic progression of cells [1]. For example, while an untransformed adherent cell undergoes a particular form of apoptosis known as anoikis after a loss of adhesion, the activity of certain oncogenes appears to be able to overcome this effect. In addition, oncogenic dysregulation, apart from a prolif-

erative hyperactivity, can induce a switch in epithelial cells from a stable adherent phenotype to a motile and invasive one. Epithelial cells establish stable contacts between adjacent cells, and between individual cells and the extracellular matrix, thus maintaining a fully polarized state. Most malignant tumors are epithelial in origin, and transition from a normal to an invasive phenotype requires drastic reprogramming on a genetic and physiological level, known as epithelial-to-mesenchymal transition [2]. This transition results in (a) both abolition and transitory activation of adhesive abilities of the cell, (b) remodeling of the actin cytoskeleton, (c) recognition of chemotactic and haptotactic cues, and (d) proteolytic processing and secretion of extracellular matrix (ECM) proteins along the trajectory. In order to perform these changes, cells need to activate a variety of signaling pathways and to change their transcriptional profiles (Fig. 1).

In order to illuminate recent advances made toward understanding the effects Rho GTPases play in this complex scenario of adhesion, migration, and invasion, we first need to explicate the specific molecular functions affected by these processes. An epithelial cell in a normal situation is laterally linked to its akin neighbors and basally rooted in the ECM, which it either produces itself or is provided by an underlying fibroblast cell layer. Cell linkage is mediated by cell–cell adhesion complexes such as tight and adherens junctions (TJs and AJs respectively), the former junctions being established more apically in the lateral membrane than the latter. Both of these junctional entities are hallmarks of fully polarized cells and indispensable for the function and maintenance of this state. Fully polarized cells are distinguished by an apical membrane domain exposed to the lumen of a particular organ or tissue, a lateral membrane domain that is linked to other cells in the layer, and a basal domain tightly linked to the ECM by focal adhesions (FA) [3].

Cell–cell and cell–matrix signaling and adhesion processes are mediated by specific cell adhesion molecules (CAMs) [4]. The principle CAM of an epithelial cell's AJs is E-cadherin. The extracellular portion of E-cadherin engages in homophilic interactions with

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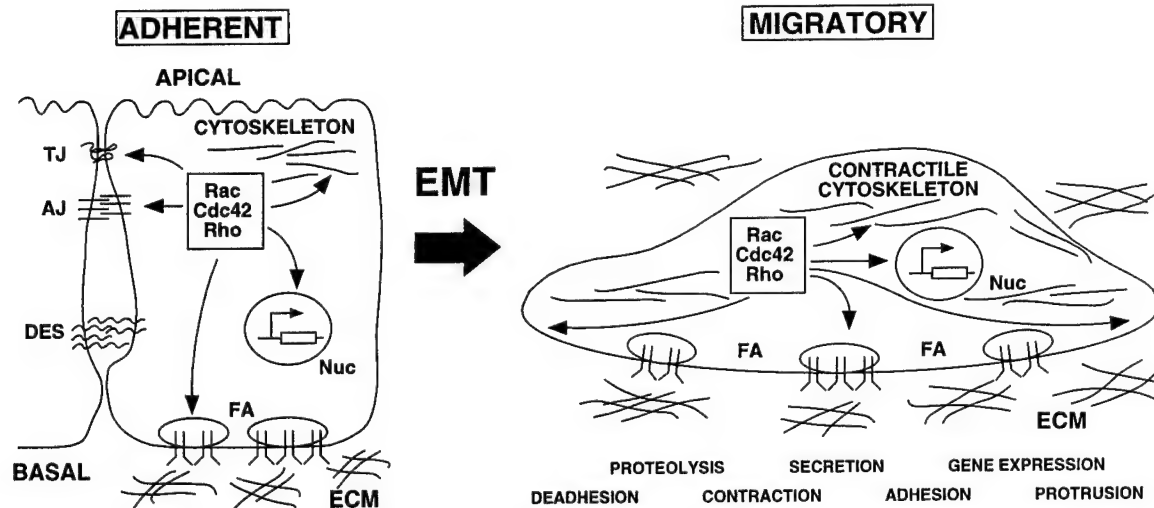


FIG. 1. Acquisition of a motile and invasive phenotype by an adherent epithelial cell. Rho GTPase function is required for both the establishment of a fully polarized state (left) and a motile phenotype upon epithelial-to-mesenchymal transition (EMT; right). Movement involves a number of individual steps which include the following: loss of cell-cell adhesion, membrane protrusions in the direction of movement, de-adhesion from the extracellular matrix in the rear and re-adhesion in front, contraction of the actin cytoskeleton in order to move the body of the cell, and active remodeling of the ECM by proteolysis and secretion. These steps also depend on alterations in the transcriptional profiling of the cell. (Nuc, nucleus; DES, desmosomes).

molecules of contacting neighbors, thus establishing an adherent layer which is subsequently sealed by the formation of TJs, in order to prevent paracellular flow of liquid and ions from one side of the sheet to the other. The intracellular portion of E-cadherin undergoes complex formation which links the protein to the actin cytoskeleton.

In addition to E-cadherin, a growing number of transmembrane proteins and cytoskeletal linkers have been found to play an important role in cell-cell and cell-matrix signaling. Transmembrane proteins transmit the effects of bound ligands through their cytoplasmic tails to intracellular signaling pathways, and these tails may also serve to anchor proteins to the actin cytoskeleton. The transmembrane component of focal adhesions consists of a heterodimeric integrin complex, consisting of an α and a β chain, which bind to ECM proteins, such as collagen, fibronectin, vitronectin, and laminin. Ligated and activated integrin dimers assemble an elaborate adhesion and signal-promoting complex at their C-terminal juxtamembrane portion [5]. In addition, growth factor receptors of the receptor tyrosine kinase (RTK) superfamily, such as receptors for epidermal growth factor (EGF) or hepatocyte growth factor/scatter factor (HGF/SF), as well as seven-transmembrane G-protein-coupled receptors (GPCRs), such as the lysophosphatidic acid (LPA) receptor, mediate chemotactic cues [6, 7].

Rho family GTPases, including Rho, Rac, and Cdc42, have been found to mediate all of the above processes in various ways [8, 9]. Rho GTPases are required for the assembly of functional cell-cell contacts (AJs and TJs) and for their disassembly during motility. Rho

GTPases control individual aspects of the actin cytoskeleton through distinct effector proteins. During the movement of the cell through the ECM, the individual steps are coordinated in a highly complicated and not yet fully understood manner. This process requires a defined level of activity and proper spatio-temporal regulation of each of the Rho GTPases, as well as cross-talk between them.

Like all GTPases, Rho GTPases act as molecular switches which cycle between an inactive GDP-bound and an active GTP-bound state, and the ratio between these two forms is dependent upon the activity of regulatory factors. GTPase-activating proteins (GAPs) promote the inactive state of a GTPase by increasing the GTPase's intrinsic rate of nucleotide hydrolysis, while guanine nucleotide dissociation inhibitors (GDIs) interfere with both the exchange of GDP for GTP and the hydrolysis of bound GTP. Guanine nucleotide exchange factors (GEFs) promote the active GTP-bound state and tether the GTPases to specific subcellular locations in order to generate an active signal. GTP-bound GTPases then associate with downstream effectors that trigger particular cellular responses [10, 11]. While dominant active mutants such as Cdc42V12, RacV12, and RhoV14 correspond to the permanently GTP-bound state, dominant negative mutants (Cdc42N17, RacN17, and RhoN19) bind more tightly to GEFs than the wild-type GTPases, but do not bind to effector proteins [12].

In this article, we summarize some intriguing recent findings that provide molecular insights into how signaling by Rho GTPases results in a motile and invasive

phenotype, focusing our attention on epithelial cells in a malignant context.

RAC AND CDC42 SIGNALING

The Rho GTPases have been implicated in a wide variety of cellular processes, including cytoskeletal organization, cell adhesion to the substratum, cell polarity, and transcriptional activation. They are best known for their distinct effects on the actin cytoskeleton [9]. In classical Swiss 3T3 fibroblast studies, activation of Cdc42 leads to the formation of filopodia, Rac results in the formation of lamellipodia and membrane ruffling, and Rho causes the formation of stress fibers [13]. The cytoskeletal rearrangements caused by activation of Rho GTPases play a key role in the process of cell motility. It is the adhesion, subsequent loss of attachment, and re-adhesion of lamellipodia and filopodia at a cell's leading edge to the substratum which result in the coordinated and polarized movement of a cell (Fig. 1) [14–16]. On the other hand, Rac and Cdc42 also play a role in cell–cell adhesion in epithelial cells in addition to their effects on the actin cytoskeleton and motility. Expression of a dominant active form of Rac in MDCK cells or keratinocytes leads to an increase in E-cadherin complex members and filamentous actin (F-actin) at cell–cell contacts, while a dominant negative mutant was found to disrupt cell–cell adhesions [17–19]. In addition, recent studies have shown that Cdc42 plays an important role in establishing the initial polarization of epithelial cells, ultimately resulting in the formation of proper cell–cell adhesions, the disassembly of which is required for motility. For example, the introduction of a dominant negative mutant form of Cdc42 into Madine–Darby canine kidney (MDCK) cells results in the selective depolarization of basolateral membrane proteins due to inhibition of membrane transport [20]. Expression of a dominant active Cdc42 mutant in MDCK cells increased AJs and prevented cellular migration induced by HGF/SF [21]. As further discussed below, it is important to note that effects of Rac and Cdc42 in a cell are cell-type, stimulus, substratum, and concentration dependent. Given the importance of Rac and Cdc42 in the cellular events described above, perturbation of the natural balance of these GTPases in a cell may ultimately lead to phenotypes of invasion and metastasis.

The process of invasion involves a number of discrete steps leading to the attainment of this state. It involves alterations in cell–cell and cell–substrate adhesion, remodeling of the ECM, reorganization of the actin cytoskeleton, and an increase in cell motility. The first evidence of a role for Rac in invasion was obtained when the Rac-specific GEF Tiam-1 (T-lymphoma invasion and metastasis) was identified in a retroviral insertional mutagenesis screen. Virus-infected T-lym-

phoma cells were selected repeatedly for *in vitro* invasion through a layer of fibroblasts and the proviral insertions in invasive clones were used to identify the Tiam-1 gene [22]. Subsequently, Rac, and later Cdc42, were shown to also confer an invasive potential to these T-lymphoma cells [23, 24]. Tiam-1 becomes activated upon localization to the plasma membrane, presumably via binding of its PH domain to the lipid products of PI3-K [25, 26]. Recent experiments have shown that activation of Tiam-1 increases the motility and *in vitro* invasion of a murine breast cancer epithelial cell line, SP-1, by mediating the effects of hyaluronic acid (HA) downstream of the HA receptor, CD44, and the cytoskeletal protein ankyrin [27, 28]. However, in contrast to the above studies, Tiam-1 has been shown to inhibit migration of NIH3T3 fibroblasts through fibronectin-coated filters [29] and to abrogate HGF/SF-induced scattering in Ras-transformed MDCK cells [30]. These effects have since been shown to be ECM dependent. On fibronectin and laminin, Tiam-1/Rac signaling inhibits invasion, while on collagen it favors motility [26].

Further evidence for involvement of Rac and Cdc42 in cellular processes pertaining to motility and invasion has since been provided. For example, expression of the laminin-receptor $\alpha 6 \beta 4$ integrin in the breast carcinoma cell line MDA-MB-435 promotes invasiveness in a Rac and PI3-kinase-dependent manner [31]. Also, activated mutant forms of Rac and Cdc42 in T47D breast carcinoma epithelial cells induce invasion through a collagen matrix. Surprisingly, however, this invasion can be blocked by PI3-K inhibitors, suggesting that PI3-kinase acts downstream of Rac and Cdc42 in this system [32]. More recent evidence for Rac in cell motility comes from fibroblasts deficient for the tumor suppressor gene Pten. *Pten*^{-/-} cells are more motile and contain higher levels of Rac*GTP and Cdc42*GTP than wild-type cells, and the motile behavior of these cells can be suppressed by dominant negative mutant forms of Rac and Cdc42 [33]. Since Pten is a lipid phosphatase which hydrolyzes PI(3,4,5)P₃ generated by PI3-K [34, 35], these experiments not only provide evidence for Rac and Cdc42 in cell motility, but also implicate PI3-kinase in this process.

Finally, numerous groups have established a role for Rho GTPases in integrin-mediated motility. Upon binding to ECM proteins, integrins assemble a focal adhesion complex, containing cytoskeletal proteins such as talin, α -actinin, vinculin, and paxillin, as well as focal adhesion kinase (FAK). Activated FAK then recruits Src family kinases, such as Src and Fyn, to the FA, resulting in the phosphorylation of paxillin [5]. Expression of dominant negative forms of the Rho GTPases has been shown to interfere with these processes [36]. Furthermore, a complex of adaptor proteins has been shown to mediate integrin-dependent signaling

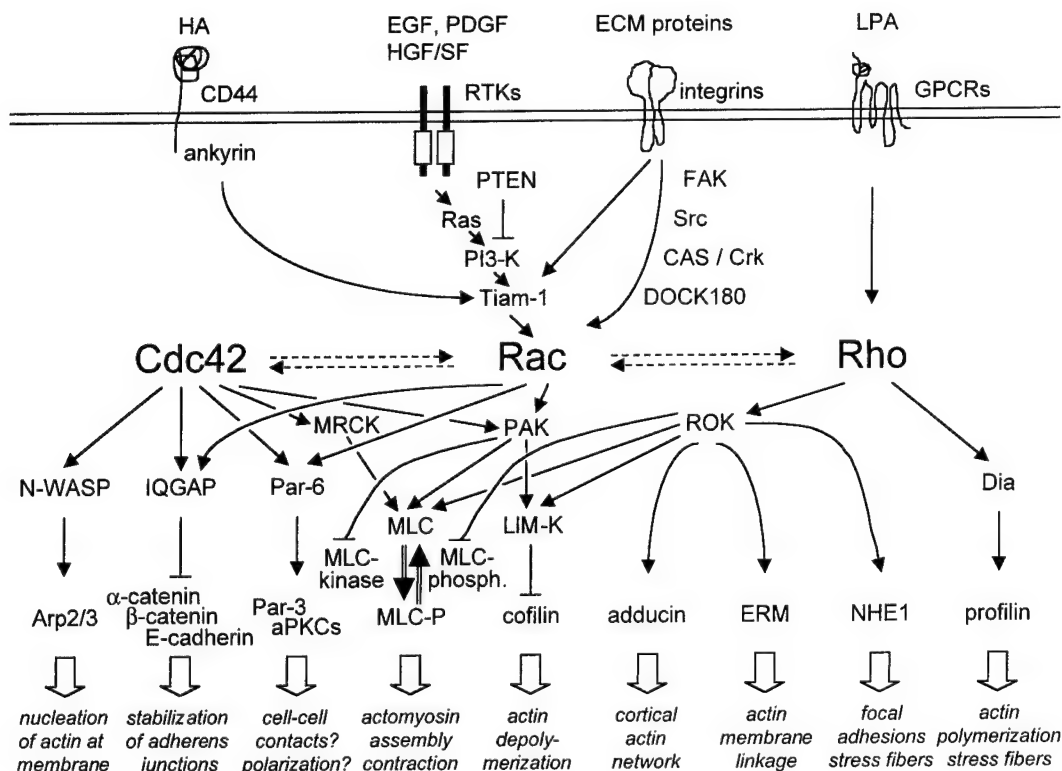


FIG. 2. Regulators and effectors of the Rho GTPases. Transmembrane receptors and their ligands (top) activate Rho GTPases through GEFs such as Tiam-1 or adaptor proteins. Activated Rho GTPases bind to and activate protein kinases, such as members of the MRCK, PAK, or ROK families, or to scaffolding proteins. These effector proteins then interact with several proteins with distinct effects on the actin cytoskeleton and cellular morphology (bottom). The spatiotemporal regulation of the individual interactions results in the overall control of motility. The cross-talk between the Rho GTPases is indicated by dashed arrows. Note that in particular the equilibrium between myosin light chain (MLC) and phospho-MLC (double arrows) is tightly regulated. See text for details.

upstream of Rac. FAK and Src kinases are involved *in vivo* in the phosphorylation of the adaptor protein CAS, and phosphotyrosine residues of CAS are then recognized by the SH2 domains of the Crk proto-oncogene. When expressed in COS cells, CAS and Crk cooperate in stimulating migration toward vitronectin, which is abrogated by dominant negative RacN17 [37]. In addition, Crk can bind to the docking protein DOCK180 [38] (which was shown to activate Rac [39]), and DOCK180 can enhance the migration-promoting potential of CAS and Crk [40].

EFFECTORS OF RAC AND CDC42

Numerous effectors of Rac and Cdc42 which mediate some of the cellular activities described above have been identified (Fig. 2). Some effectors have been found to specifically mediate cell motility, whereas others play a more prominent role in cell adhesion, thus further suggesting involvement of Rho GTPases in these processes under normal and aberrant conditions. As discussed below, WASP and MRCKs serve as Cdc42-specific effectors crucial to actin organization and for-

mation of filopodia and thus a motile phenotype. In addition, members of the p21-activated kinase family (referred to as PAK) are protein kinases downstream of Rac and Cdc42 which play an important role in cytoskeletal-mediated changes which affect motility. The scaffolding proteins IQGAP and Par-6, which can both be activated by either Cdc42 or Rac, promote cell polarization and contribute to cell-cell adhesion. Perturbation of these molecules, and thus cell-cell contacts, suggests that they may promote motility through disruption of the normal organization of neighboring cells.

As mentioned above, the ubiquitously expressed and neuronally enriched N-WASP is a scaffolding protein specific for Cdc42 [41–43]. Moreover, it binds to the Arp2/3 complex of actin-nucleating proteins crucial for the localized assembly of an actin network within filopodia [44, 45, 114, 115]. It was recently shown that both N-WASP and the Arp2/3 complex are necessary for Cdc42 to trigger actin filament assembly, and reconstitution experiments using purified proteins *in vitro* have shown that maximal actin polymerization requires not only Arp2/3, N-WASP, and Cdc42*GTP, but also the phospholipid PI(4,5)P₂ [46]. Thus, through

proper filopodia formation, N-WASP may promote cellular motility.

MRCKs α and β are Cdc42-specific effectors which can phosphorylate myosin light chain (MLC) via a ROK-like kinase domain [47]. Phosphorylation of MLC is required for the assembly of actomyosin complexes and their contraction, and it is the equilibrium between MLC and phospho-MLC, mainly regulated by the opposing effects of MLC-kinase (MLCK) and MLC-phosphatase (Fig. 2), which plays a pivotal role in generating force to translocate the cell body during movement. In addition, overexpression of MRCK α with Cdc42 synergizes to promote filopodia formation, while a MRCK α kinase-deficient mutant inhibits the formation of Cdc42-induced filopodia [47]. Therefore MRCKs appear to play an important role in cytoskeletal organization and contraction, which contribute to the process of migration.

PAK is a protein kinase downstream of Rac and Cdc42 [48] which plays a crucial role in actin dynamics and adhesion, and there are at least three ways through which it exerts its physiological effects. These include phosphorylation of MLCK and LIM-kinase, as well as focal complex disassembly. First, PAK has been demonstrated to phosphorylate and hence inactivate MLCK [49], resulting in a decrease in MLC phosphorylation. Thus, inactivation of MLCK results in the disassembly of stress fibers and focal adhesions, suggesting potential for motility. Indeed, decreased phosphorylation of MLC in kidney cells expressing activated PAK results in a decrease in cell spreading on fibronectin [49]. Also, invasion by highly invasive breast cancer cells, MDA-MB-435, into a gel matrix substrate is abrogated by expression of a kinase-defective PAK mutant [50]. However, PAK has also been observed to phosphorylate MLC in human microvascular endothelial cells (HMEC-1), and in this case, it is presumed that while PAK is not required for the formation of lamellipodia, it still plays a role in cell adhesion and contraction, thus contributing to cell motility [51]. In addition, an increase in MLC phosphorylation was also found in PAK-expressing NIH3T3 fibroblasts, resulting in increased directionality of haptotactic movement through a collagen gradient [52]. Secondly, PAK may control the actin cytoskeleton through the phosphorylation and subsequent activation of LIM-kinase. Phospho-LIM-kinase further phosphorylates and thereby inactivates the actin-depolymerizing protein cofilin, thus inhibiting actin depolymerization when Rac is activated and causing extreme membrane ruffling suggestive of a motile phenotype [53–55]. Thirdly, pathways involving PAK have become even more intricately interwoven with the discovery of PIX, also known as Cool [56, 57]. Manser *et al.* have provided data supportive of PIX being a Rac GEF [56]. PIX interacts with PAK and causes it to localize to

focal contacts (FCs) which are integrin-dependent sites linked to the actin cytoskeleton [56]. Dissolution of FCs is necessary in order for a cell to migrate, and this is believed to occur via interaction of PIX with the G-protein-coupled receptor kinase-interacting protein (GIT1), which when overexpressed causes a loss of paxillin from FCs and promotes migration [58]. GIT1 also directly couples FAK, known to be involved in FC turnover, to FCs. Interaction of PAK with PIX leads to an increase in PAK's activity [56], and interaction of PIX with PAK allows for the formation of a trimeric GEF/GTPase/effector complex with a built-in positive feedback loop and mutual regulation of activity [116]. It also links PAK to FAK through GIT1, promoting FC turnover and thus motility [58]. Altogether, the Rac effector PAK appears to play a vital role in promoting Rac-dependent motility through a number of different regulatory mechanisms and signaling cascades.

The IQGAP1 and IQGAP2 scaffolding effectors of Cdc42 and Rac [59, 60] may regulate cell–cell adhesion through actin polymerization and sequestration of the adhesion complex molecule β -catenin. *In vitro*, IQGAP oligomerizes and cross-links F-actin [61] and it has been found to complex with Cdc42 and F-actin *in vivo* [60]. In addition, one study has shown that the IQGAP protein also competes with α -catenin for binding to β -catenin, thus preventing attachment of the E-cadherin/ α -catenin/ β -catenin complex to the actin cytoskeleton and thereby disrupting cell–cell contacts. Furthermore, the same study showed that Cdc42* GTP prevented IQGAP1 from disrupting cell–cell contacts, possibly by binding IQGAP1, and preventing it from associating with β -catenin [62]. Hence, IQGAP may provide a molecular mechanism by which Rac and Cdc42 can regulate cell–cell adhesion.

Another scaffolding protein, Par-6, is the most recently identified shared Cdc42 and Rac effector. Par-6 was identified using activated Cdc42 and TC10 mutants as baits in yeast two-hybrid screens [63, 64]. Par-6 binds to a second scaffolding protein, Par-3, and both Par-6 and Par-3 bind independently to atypical protein kinase C (aPKC) isoforms [63–65]. Most interestingly, endogenous Par-3 localizes to TJs in MDCK cells, while overexpression of Par-6 or the N-terminal portion of Par-3, the region responsible for Par-6 interaction, disrupts their formation. In addition, high expression levels of full-length Par-3 cause cell spreading beneath surrounding untransfected cells, while still maintaining a small area of tight-junctional attachment [64]. Thus we can speculate that aberrant, hyperactive Cdc42 signaling may facilitate a motile phenotype by disrupting cell–cell tight-junctional contacts. The Par-6/aPKC complex was also shown to be downstream of Rac1 and to potentiate the transforming effect of Rac1 [63]. This may be explained by a loss of cell–cell contacts as observed in the above study

[64], which could result in a loss of contact inhibition and excessive growth.

Alterations in cell morphology, such as reorganization of the actin cytoskeleton by Rac and Cdc42 above, are initiated when a cell receives a signal from its environment, resulting in a signaling cascade from receptor to nucleus, in which changes in gene expression ultimately take place. Changes in expression of proteins such as matrix metalloproteinases, including collagenase-1 (CL-1) and stromelysin-1, are vital to effecting cytoarchitectural rearrangements, since they are crucial for remodeling the ECM [66]. Kheradmand *et al.* studied the role Rho GTPases play in integrin-induced expression of CL-1 in rabbit synovial fibroblasts. They found that an increase in CL-1 resulting from disruption of $\alpha 5 \beta 1$ integrin adhesion was dependent on Rac1, since a dominant negative form of Rac1 blocked expression of CL-1. In addition, an activated mutant form of Rac1 was sufficient to increase CL-1 expression, and this induction resulted in the generation of reactive oxygen species and was dependent on the nuclear factor κB and interleukin-1, which acts in an autocrine manner to induce CL-1 [67]. In addition, Rac was also found to play a role in AP-1 regulation of a multigenic invasion program [68]. Interestingly, promoters of metalloproteases contain a binding site for AP-1 transcription factors, which are composed of heterodimers of the Fos and Jun protein families and are regulated by MAPK pathways [69]. In a human squamous cell carcinoma line, which generally exhibits an invasive phenotype upon EGF stimulation, it was found that a dominant negative mutant form of c-Jun is capable of preventing translocation of Rac to the membrane, thereby preventing its activation. In addition, expression of this mutant also disrupted lamellipodia formation and membrane ruffling, as well as motility and invasion [68]. From these studies, we can conclude that the physiological effects of Rac discussed above are dependent not only upon protein-protein interactions and posttranslational modifications, but also upon protein expression, and that invasion is dependent upon an intricate reprogramming of the cell.

It is evident from the studies described above that both Rac and Cdc42 play an important role in motility and adhesion, utilizing a plethora of signaling molecules. In addition, there is evidence that they play a role in invasion and thus contribute to an oncogenic state. It is noteworthy that in the process of invasion, events distinct from motility may also contribute to an invasive phenotype [70]. It appears that fine-tuned regulation of these GTPases and their interacting molecules is necessary to maintain a healthy cell state, and their effects within a cell are determined by numerous factors, such as concentration, cell type, stimulus, substrate, localization, and timing.

RHO SIGNALING

Rho was first implicated in the formation of stress fibers and FA complexes [13], resulting in actomyosin assembly and contractile tension, two processes necessary for the forward movement of a cell. Rho has since been shown to play an important role in cell-cell adhesion. In particular, inactivation of RhoA by C3 transferase, which ADP-ribosylates the Asn41 residue, disrupts organization of actin filaments at cell-cell contacts, thus inhibiting the proper formation of both AJs and TJs [17, 18, 71, 72]. For example, in normal mammary epithelial cells, MCF10 cells, inhibition of Rho by C3 transferase disrupts E-cadherin cytoskeletal links in AJs and blocks the formation of new AJs [72]. Whereas Rho is required for the assembly of AJs, Zhong *et al.* further demonstrated that high levels of activated Rho in Ras-transformed cells contribute to the fibroblastic phenotype of these Ras-transformed epithelial cells [72]. As detailed below, the balance between contractility and cell-cell adhesion, in addition to GTPase concentration, will determine whether a cell takes on a static or a motile phenotype. Depending on different variables, such as cellular context, stimulus, and extracellular matrix, Rac and Cdc42 activation have been reported to either promote or antagonize Rho's function. In Swiss 3T3 fibroblasts, the Rho GTPases have been placed in a hierarchical order in which Cdc42 activates Rac, and Rac activates Rho ([13]; see also [8]), while in N1E-115 neuroblastoma and MDCK cells, constitutively activated Rac down-regulates Rho [73, 74]. Therefore, cross-talk and a balance between individual Rho GTPases, as well as varying internal and external cellular factors, may result in a particular response [75].

There are several lines of evidence that directly link Rho to the acquisition of migratory, invasive, and metastatic phenotypes. RhoA has been implicated in an $\alpha 6 \beta 4$ integrin-mediated pathway which is regulated by cAMP, and, somewhat surprisingly, this pathway results in the formation of lamellipodia and migration in Clone A colon carcinoma cells. Expression of a dominant negative form of RhoA resulted in the attenuation of membrane ruffling and lamellipodia formation, as well as migration, and RhoA localization to lamellipodia was blocked by inhibiting phosphodiesterase activity and enhanced by inhibiting cAMP-dependent protein kinase activity [76]. Furthermore, activation of Rho, such as by LPA, and stimulation of the actomyosin system have been associated with the transmigration of tumor cells. For example, in a metastasis assay, NIH3T3 fibroblasts expressing an activated mutant form of RhoA were injected into the tail vein of nude mice and resulted in metastasis in the lung [77]. Furthermore, in the absence of serum, activated RhoA has been found to promote invasion of cultured rat MM1

hepatoma cells through a mesothelial cell monolayer [78], and cells implanted into the peritoneal cavity resulted in invasion of the peritoneum and established tumors [78, 79]. Finally, a RhoC isoform was recently identified in a microarray-based screen for genes over-expressed in metastatic melanomas. Involvement of RhoC in metastasis was confirmed by transducing poorly metastatic A375P cells with RhoC and noting enhanced metastasis when reintroduced into mice. These cell lines were also tested for migratory and invasive potential, and it was found that introduction of RhoC into the A375P cell line resulted in an increase in migration and invasive behavior [80].

EFFECTORS OF RHO

Members of at least two protein families appear to be required for Rho-induced assembly of stress fibers and FAs, the Rho-associated kinases (henceforth referred to collectively as ROK) and the Dia members of the formin homology family. These include the Rho effectors p164ROK α (Rho-associated kinase), p160ROK β (Rho-associated coiled-coil containing protein kinase or ROCK) [81–83], Dia1, and Dia2 [84, 85]. Activation of ROK appears to be necessary, but is not sufficient, for stress fiber formation. Inhibition of ROK, using the inhibitor Y-27632, prevents stress fiber formation [86], while a constitutively activated mutant of ROK α merely promotes the formation of stellate actomyosin filaments, not stress fibers, in MDCK and HeLa cells [87, 88, 100]. It appears that synergy between ROK and Dia is required for proper formation of stress fibers [89, 90, 100]. Dia may contribute to stress fiber formation through its interaction with profilin, a G-actin binding protein which promotes actin polymerization and organization of actin filaments into stress fibers [84, 85, 89].

At least six substrates of ROK may also play a role in actin cytoskeletal reorganization and thus contribute to adhesion (see Fig. 2). As discussed below, these include MLC, the myosin-binding subunit of MLC phosphatase, LIM-kinase, adducin, the ERM (ezrin/radixin/moesin) family of proteins, and a Na⁺/H⁺ exchange protein (NHE1). Rho-kinase members encode for Ser/Thr kinases and have been demonstrated to phosphorylate and thus influence the activity of these molecules. The effects of three of these ROK effectors, namely MLC-phosphatase, MLC, and LIM-kinase, are best known and have been found to play an important role in driving ROK's physiological function on the actin cytoskeleton. ROK has been shown to phosphorylate, and thereby inactivate, MLC phosphatase, thus resulting in an increase in phosphorylated MLC [91, 92]. In addition, ROK can also phosphorylate MLC directly [93]. An increase in phosphorylated MLC results in enhanced actomyosin assembly and therefore

an increase in stress fiber formation, adhesion, and contractility. This activity of ROK opposes inactivation of MLCK by Rac and Cdc42 via PAK (see above) and may explain apparent antagonistic effects between both Cdc42 and Rac and Rho in some cell types [94]. ROK also causes stabilization of filamentous actin through its phosphorylation of LIM-kinase, which subsequently phosphorylates and thus inactivates cofilin [95]. As mentioned above, LIM-kinase has also been shown to be phosphorylated by PAK, thus revealing yet another potential point of convergence for Rac and Rho pathways.

The ROK effectors adducin and the ERM family of proteins provide more direct regulation of the actin cytoskeleton. ROK has been shown to phosphorylate adducin [96, 97], which, together with spectrin, is an important component of the cortical actin network underlying the plasma membrane [98]. ROK-phosphorylated adducin interacts with F-actin, and its localization suggests that it may be important in the migration of a cell. In tetradecanoylphorbol 13-acetate or HGF/SF-stimulated MDCK cells, phosphoadducin localizes to membrane ruffles, and ROK-phosphorylated adducin localizes to the leading edge of migrating NRK49F fibroblasts in a wound-healing assay [97]. Furthermore, introduction of nonphosphorylatable adducin into MDCK and NRK49F cells inhibited membrane ruffling and migration in assays similar to the ones mentioned above, as did a dominant negative ROK mutant and C3 exoenzyme [97]. Finally, a constitutively active adducin mutant overcame inhibition of membrane ruffling by a dominant negative ROK mutant [97]. ROK can also phosphorylate ERM proteins important for linking actin filaments to the plasma membrane, and in this manner may affect cell adhesion and motility as well [99; however, see also 101]. Interestingly, more recently the TSC1 tumor suppressor hamartin has been demonstrated to control cell adhesion to the cell substrate through the ERM family of actin-binding proteins and RhoA [102]. Finally, a sixth ROK substrate, namely NHE1, is a ubiquitous Na⁺/H⁺ exchange protein which potentiates stress fiber formation [103].

ROK recently also has been found to increase activity of phosphatidylinositol 4-phosphate 5-kinase (PI(4)P5-K) and subsequently increase phosphatidylinositol 4,5-bisphosphate, PI(4,5)P2, levels in HEK-293 epithelial cells [104]. PI(4)P5-K is a kinase known to generate the lipid signaling molecule PI(4,5)P2, which is involved in the Rho signal transduction pathway [105]. In particular, PI(4,5)P2 has been found to be necessary for stress fiber formation, as well as actin polymerization and perhaps FA assembly [105, 106]. Through its interaction with these different substrates named above, ROK influences the cytoskeletal structure of a cell and promotes the formation, organization,

and contraction of actin filaments in order to ultimately effect functions of Rho known to be involved in cell morphology.

Evidence exists which suggests that ROK plays a role in tumor cell invasion and metastasis. As mentioned above, activated RhoA has been found to promote invasion of cultured rat MM1 hepatoma cells through a cell monolayer [78]. In a similar assay, a dominant active form of p160ROK was also found to confer invasive potential to MM1 cells in the absence of serum and activation of Rho, while a kinase-defective ROK decreased invasive activity. In addition, the ROK-specific kinase inhibitor Y-27632 blocked Rho-induced actomyosin activation, as indicated by inhibition of MLC phosphorylation, as well as the invasive phenotype. Furthermore, continuous exposure to this inhibitor reduced the dissemination of MM1 cells implanted into the peritoneal cavity of syngeneic rats, as well as incidences of tumor nodules [79]. Further evidence implicating ROK in metastasis comes from the study of human cell lines found to play a role in intrahepatic metastasis of human hepatocellular carcinoma (HCC) [107]. When highly intrahepatic metastatic HCC cell lines were examined *in vitro*, they were found to exhibit a high degree of motility which was upregulated by LPA and inhibited by C3 exoenzyme, suggesting that activation of the Rho signaling pathway is necessary for motility in these aberrant cells. In addition, stable transfection of these cell lines with dominant negative and active forms of p160ROK also affected motility *in vitro*. Dominant negative transfectants exhibited a decrease in motility, while dominant active transfectants showed an increase in motility even in the absence of serum. Also, orthotopic implantation of intrahepatic metastatic HCC cells stably expressing a dominant negative mutant form of ROK resulted in a decrease in the metastatic ability of this cell line, suggesting that ROK is necessary for metastasis in this system [107].

As in the case of Rac, drastic changes in cellular morphology, which result from activation of Rho, most likely require extensive transcriptional reprogramming of the cell in addition to posttranslational modifications. Serum response factor (SRF) is a MADS box transcription factor which regulates cellular immediate early genes and skeletal, smooth, and cardiac muscle genes [108, 109]. It has been shown that serum and LPA-induced SRF activation is dependent upon RhoA, as is constitutively active SRF at muscle-specific promoters [110–112]. An *in vivo* screen for SRF activators identified LIM-kinase-1 as a strong SRF activator [113]. While LIM-kinase-1 is not essential for serum-induced activation of SRF, it has been shown to cause F-actin aggregation, which is dependent upon phosphorylation of cofilin, and it is regulation of actin treadmill which actually activates SRF [113]. In addition,

activated forms of mDia1 and mDia2 are both capable of activating SRF, while microinjection of an anti-mDia1 antibody into serum-deprived cells containing a stably integrated SRE-regulated reporter gene inhibited SRF activation by serum [117]. These studies therefore provide a direct link between Rho-induced actin-cytoskeletal rearrangements and gene transcription.

It is evident from the above studies that Rho and numerous downstream molecules play an important role in contractility and adhesion under normal conditions. In addition, there is growing evidence that Rho and its effector, ROK, are directly involved in invasion and metastasis. The ROK substrate adducin has been directly implicated in migration. It appears that fine-tuned regulation of Rho and related molecules is vital for a healthy cell state, and their effects within a cell are determined by numerous factors, such as concentration, cell type, stimulus, substrate, localization, and timing.

CONCLUSIONS

The findings referred to in this article provide evidence for participation of Rho GTPases in the migration and invasion of deregulated cells. Many of the essential functions performed by Rho proteins on cytoskeletal and transcriptional levels can be oncogenically corrupted and may result in a typical tumor phenotype. The fact that a number of oncogenes encode exchange factors for the small Rho GTPases, Rho, Cdc42, and Rac, in addition to the recent observation that RhoC is transcriptionally upregulated under invasive conditions, further reinforces this notion.

Since the activating mechanisms and molecular pathways of these molecular switches are diverse in nature and context, and the effects probably even more varied, it is to be expected that there will be much more to learn. Discrepancies as to the function of a molecule often arise due to cell type, stimulus, substrate, timing, protein localization, and experimental design differences. New molecules upstream and downstream of those already implicated in tumor invasion and metastasis will undoubtedly be identified in the future, further elucidating the mechanisms by which these processes are carried out. Furthermore, identification of the spectrum of genes instrumental in the materialization of a fully invasive behavior, which are transcriptionally activated by Rho GTPases, will also shed light on cancerous states.

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[11] Ras and Rap1 Interaction with AF-6 Effector Target

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Introduction

The Rap types of small GTPases are members of the Ras superfamily and are the molecules that show the most identity with the oncogenic Ras proteins. Whereas the interaction of activated Ras proteins with their downstream effectors Raf, Ral guanine nucleotide dissociation stimulator (RalGDS), and phosphatidylinositol 3-kinase (PI3K) led to a fairly defined

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METHODS IN ENZYMOLOGY VOL. 332

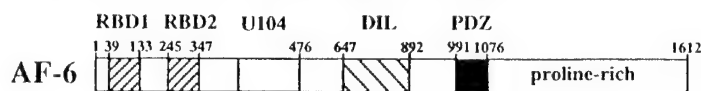


FIG. 1. Schematic representation of AF-6.

picture, the role of Rap1 is as yet poorly understood.¹ Besides its ability to bind to Raf and RalGDS, two-hybrid and *in vitro* experiments suggest that still another molecule, namely AF-6 ALL1-fused gene on chromosome 6, might serve as a relevant target. Both two-hybrid and kinetic studies suggest a strength of interaction that exceeds the one exerted by Ras-AF-6 complexes, which originally led to the identification of AF-6.^{2,3} AF-6 has also been described as a fusion partner for ALL1 in acute lymphoblastic leukemias.⁴ The AF-6 protein contains a combination of interesting homology regions.⁵ At its very NH₂ terminus reside two putative Ras/Rap 1-binding motifs, followed by U104 and DIL motifs, domains that are found in the head portions of microtubule- and actin-based motor proteins, respectively. Located further to the COOH terminus is a PDZ domain succeeded by proline-rich clusters, which may function as docking sites for other molecules (Fig. 1).

In this chapter, we outline the methods that allowed us to investigate the physical interaction between Ras/Rap1 and AF-6, namely two-hybrid analyses, kinetic and thermodynamic studies, and *in vivo* studies utilizing retrovirally engineered cell lines.

AF-6 resides in cell-cell adhesion complexes and could provide the molecular link between the activity of Ras/Rap1 proteins and their effects on intercellular adhesion.

Use of Yeast Two-Hybrid System to Evaluate Ras/Rap1 Interaction with AF-6

Principle

One approach we took to examine the interaction between Ras/Rap1 and AF-6 consists of a two-hybrid interaction trap assay. This system is a

¹ J. L. Bos, *EMBO J.* **17**, 6776 (1998).

² L. Van Aelst, M. A. White, and M. H. Wigler, *Cold Spring Harbor Symp. Quant. Biol.* **59**, 181 (1994).

³ M. Kuriyama, N. Harada, S. Kuroda, T. Yamamoto, M. Nakafuku, A. Iwamatsu, D. Yamamoto, R. Prasad, C. Croce, E. Canaani, and K. Kaibuchi, *J. Biol. Chem.* **12**, 607 (1996).

⁴ R. Prasad, Y. Gu, H. Alder, T. Nakamura, O. Canaani, H. Saito, K. Huebner, R. P. Gale, P. C. Nowell, K. Kuriyama, Y. Miyazaki, C. M. Croce, and E. Canaani, *Cancer Res.* **53**, 5624 (1993).

⁵ C. P. Ponting and D. R. Benjamin, *Trends Biochem. Sci.* **21**, 422 (1996).

genetic method that allows the determination of physical complexes between two proteins within yeast cells.⁶ These proteins are expressed as hybrid proteins, one fused to a DNA-binding domain and the other fused to a transcription-activating domain. If the two proteins associate, a functional transcription factor is reconstituted and a reporter gene is transcribed. Several versions of the two-hybrid system exist; they commonly involve DNA-binding domains derived from Gal4 (GBD) or LexA (LBD) and activation domains from Gal4 (GAD) or VP16 transcriptional activators.^{6,7} Numerous yeast strains with different reporter genes for both systems have been constructed, as well as variations of the original two-hybrid system, including a reverse one- and two-hybrid system, and a three-hybrid system. The more recently constructed yeast strains, with multiple reporter genes harboring independent promoters, offer a major advantage because they increase specificity, thus limiting false positives. A detailed description can be found in Vidal and Legrain⁸ and in Brent and Finley.⁹ The choice of using either the Gal4- or LexA-based system will be dependent on the nature of the protein of interest.¹⁰ In our studies directed to assess the interaction between Ras/Rap1 and AF-6, we found that both Gal4- and LexA-based systems can be used. The use of the LexA-based system is presented here. The application of the two-hybrid system allowed us to assess whether the binding of AF-6 requires Ras/Rap1 to be in a GTP-bound state, to map the minimum domain of AF-6 required for interaction, and to compare binding profiles between Ras, Rap1, AF-6, and other Ras/Rap1 targets.

Materials

Yeast Strain. The yeast strain L40 (*Mata his3 Δ 200 trp1-901 leu2-3,112 ade2*) containing *HIS3* and *lacZ* as reporter genes is used for the LexA-based system.⁷

Media. YPD medium contains 10 g of yeast extract, 20 g of Bacto-Peptone (Difco, Detroit, MI), 2% (w/v) glucose, and 20 g of agar for plates, per liter. DO-Leu-Trp and DO-Leu-Trp-His media contain Bacto-Yeast nitrogen base without amino acids (0.67%, w/v), dropout mix (0.2%, w/v), and Bacto-Agar (2%, w/v). Dropout mix is a combination of all essential amino acids minus the appropriate supplement.¹⁰

⁶ C. T. Chien, P. L. Bartel, R. Sternglanz, and S. Fields, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 9578 (1991).

⁷ A. B. Vojtek, S. M. Hollenberg, and J. A. Cooper, *Cell* **74**, 205 (1993).

⁸ M. Vidal and P. Legrain, *Nucleic Acids Res.* **27**, 919 (1999).

⁹ R. Brent and R. L. Finley, Jr., *Annu. Rev. Genet.* **31**, 663 (1997).

¹⁰ L. Van Aelst, *Methods Mol. Biol.* **84**, 201 (1998).

Solutions

Lithium acetate/TE (0.1 M, pH 7.5): Combine 0.1 M lithium acetate (Sigma, St. Louis, MO), 10 mM Tris-HCl (pH 7.5), and 1 mM EDTA. Polyethylene glycol (PEG) 3300, 40% (w/v) in 0.1 M lithium acetate. Z buffer: Combine $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (16.1 g/liter), $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (5.5 g/liter), KCl (0.75 g/liter), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.246 g/liter); adjust to pH 7.0 and autoclave.

X-Gal stock solution: Dissolve 20 mg of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) (Boehringer Mannheim, Indianapolis, IN) in 1 ml of *N,N*-dimethylformamide. Store at -20° in the dark.

Z buffer/X-Gal solution: To 100 ml of Z buffer add 270 μ l of 2-mercaptoethanol and 1 ml of X-Gal stock solution.

Plasmids and Constructs. For the construction of pGAD AF-6N, pGAD AF-6-RBD1, and pGAD AF-6-RBD2: AF-6N (amino acids 1–368) is polymerase chain reaction (PCR) amplified with oligonucleotides AF6N-5' *Sal*I (5'-GGGACGTCGACTCTCGGCGGGCGGCCGTGACGAG-3') and AF6N-3' *Xho*I (5'-CGGCAGCTCGAGCTATCTCTCCTTTCCCTTGGGTGT-3') and inserted into *Xho*I-digested pGAD1318 plasmid, which is a derivative of pGADGH.¹⁰ AF-6-RBD1 (amino acids 1–140) is amplified with AF6N-5' *Sal*I and AF6RBD1-3' *Xho*I (5'-CCGCCGCTCGAGCTACTTAGGAGGAATGGC-3') as 5' and 3' oligonucleotides, respectively, and treated as described above. AF-6-RBD2 (amino acids 181–368) is amplified with AF6RBD2-5'BHI (5'-GGGCCGGATCCGCCATTCCTCTAAG-3') and AF6N-3' *Xho*I oligonucleotides and inserted into *Bam*HI-*Xho*I-digested pGAD1318 vector. For the construction of pGAD PI3K δ -RBD, PI3K δ -RBD is PCR amplified using 5' oligonucleotide PI3K δ RBD5' *Bam*HI (5'-CGGCGCGGATCCATGGCCAAGATGTGCCAATTCTGC-3') and 3' oligonucleotide PI3K δ RBD3' *Sal*I (5'-GCCGACGTCGACCTAGTTGCTCTGCTCATCCCG-3'), and digested with *Bam*HI and *Sal*I restriction endonucleases prior to ligation into *Bam*HI-*Xho*I-cut pGAD1318. pGAD1318 RalGDS-RBD is obtained in a yeast two-hybrid screen, using LBD Ha-RasV12 as bait and a Jurkat cDNA library cloned in pGAD1318. The plasmids pGADGH cRafN, LBD RasV12, and LBD RasN17 have been previously described.^{7,11} For the LBD Rap 1E63 and Rap1N17 constructs, the cDNAs are PCR amplified with pZip-EE-Rap1E63 (obtained from B. Knudsen, Cornell University, Ithaca, NY) and pGTB9 Rap1N17 (from D. Broek, USC, Los Angeles, CA) as templates, and as primers 5'-ATTTATGGATCCTCTAGAATGCGTGAGTACAAGCTA-3' and 5'-CTGACTCTCGAGCTAGAGCAGCAGACAT-

¹¹ L. Van Aelst, M. Barr, S. Marcus, A. Polverino, and M. Wigler, *Proc. Natl. Acad. Sci. U.S.A.* 90, 6213 (1993).

GATTT-3' are used. The products are digested with *Bam*HI and *Xho*I and subcloned into a *Bam*HI-*Sall*-digested pLexVJ10 plasmid.¹⁰

Protocols and Results

Analysis of Ras/Rap1-AF-6 Interactions, Using Liquid Assay for β -Galactosidase

As shown in Fig. 1, AF-6 harbors two predicted Ras-binding domains at its N terminus. To determine whether both domains are able to interact with Ras and Rap1, the RBD1, RBD2, and AF-6N domains of AF-6 are fused to the GAL4-activation domain of *GAL4*, whereas Ras and Rap1 mutants are fused to the LexA DNA-binding domain. In analogy to the well-characterized Ras-Raf interaction, we have also tested whether AF-6 requires Ras and Rap1 to be in the GTP-bound state. To this end we use constitutively active mutant forms (RasV12 and Rap1E63) and dominant negative mutant forms (RasN17 and Rap1N17) of Ras and Rap1. The Ras and Rap1 LDB fusion constructs are transformed together with either pGAD AF-6-RBD1, pGAD AF-6-RBD2, or pGAD AF-6N into the yeast strain L40, according to the protocol described below. Before performing the liquid β -galactosidase assay, we always first perform growth selection on selective medium (DO-Leu-Trp-His) and β -galactosidase filter assays (see below). The liquid culture assay allows us to compare and to quantify the strength of interactions.

Yeast Transformation. The protocol described below is a modification of the method of Ito *et al.*¹² and can be applied for both LexA- and Gal4-based systems.

1. Inoculate a single yeast colony into 10 ml of YPD and grow overnight at 30° with shaking. Transfer the overnight preculture into 100 ml of YPD and grow the yeast culture further at 30° with shaking (230 rpm) until an OD₆₀₀ of 0.5–0.8 is reached.
2. Harvest the cells by centrifugation at 1500g for 5 min at room temperature and wash them in 25–50 ml of 0.1 M lithium acetate in TE.
3. Resuspend the washed cells in 1 ml of 0.1 M lithium acetate in TE and incubate for 1 hr at 30° with shaking at 230 rpm. The yeast cells are now competent for transformation. One milliliter of cells allows for 10 transformations.
4. Add 100 μ l of competent cells for each transformation into a 1.5-ml microcentrifuge tube.

¹² H. Ito, Y. Fukuda, K. Murata, and A. Kimura, *J. Bacteriol.* **153**, 163 (1983).

5. Add the plasmid DNAs (approximately 0.5 to 2 μg) together with 100 μg of sheared, denaturated salmon sperm DNA to the competent yeast cells and subsequently add 600 μl of sterile PEG-lithium acetate solution. Mix well by inversion.

6. Incubate at 30° for 30–60 min (shaking is not required) and heat shock for 15–30 min in a 42° water bath.

7. Pellet the cells by centrifugation for 30 sec in a microcentrifuge, remove the supernatants, and resuspend the cells in 100 μl of sterile TE.

8. Plate the yeast cells on DO-Leu-Trp medium and incubate at 30° until colonies appear.

Liquid Culture Assay for β -Galactosidase. The liquid culture assay for β -galactosidase provides quantitative data for the Ras/Rap1-AF-6 interaction, allowing us to compare the strength of interactions. The assay described below quantifies the β -galactosidase enzymatic activity by measuring the generation of the yellow compound *o*-nitrophenol (ONP) from the colorless substrate *o*-nitrophenyl galactoside (ONPG).

1. Inoculate single colonies from the yeast transformants in 1 ml of selective medium (DO-Leu-Trp) and grow them overnight. The next day, dilute the cells 5- to 10-fold in 5 ml of fresh DO-Leu-Trp medium and incubate them further until an OD₆₀₀ of approximately 0.8 to 1 is reached. Record the OD₆₀₀ for 1-ml samples of each culture.

2. Transfer 1-ml aliquots (in triplicate) to 12 × 75-mm polypropylene tubes and pellet the cells by centrifugation. Add 1 ml of Z buffer to the cells. Include a control with Z buffer alone.

3. Add 50 μl of CHCl₃ and 50 μl of 0.1% (w/v) sodium dodecyl sulfate (SDS) to the tubes and vortex vigorously for 10 sec to resuspend the cells.

4. Prewarm the samples to 30° for 5 min and then add 0.2 ml of ONPG solution to each tube. Vortex and incubate the reactions at 30°, until color develops (between 10 min and 6 hr). Stop the reaction by adding 0.5 ml of Na₂CO₃ (1 M) followed by brief vortexing. Centrifuge the samples for 10 min (3500 rpm at room temperature) and remove 1 ml of each sample to a disposable cuvette. Measure the OD at 420 nm against the blank.

5. Calculate the β -galactosidase activity by using the following equation: Activity (in Miller units) = $1000[(\text{OD}_{420} - \text{OD}_{\text{blank}})]/(tV\text{OD}_{600})$, where *t* is time of incubation, *V* is volume (ml) of initial cells aliquoted, and OD₆₀₀ is cell density of the culture.

The results obtained in the β -galactosidase liquid culture assay are shown in Table I. We noticed that AF-6N and the first domain (RBD1), but not the second domain (RBD2), were able to bind to Ras and Rap1. Furthermore, while both activated mutant forms of Ras and Rap1 interact

TABLE I
INTERACTION BETWEEN Ras/Rap1 AND AF-6

LBD fusion	β -Gal activity of GAD-fused AF-6 domains (Miller units) ^a		
	AF-6N	AF-6-RBD1	AF-6-RBD2
RasV12	99 \pm 1.6	210 \pm 1.8	0.7 \pm 1.4
RasN17	0.6 \pm 1.4	0.9 \pm 1.4	0.6 \pm 1.1
RapE63	120 \pm 1.7	350 \pm 1.9	1.2 \pm 1.1
RapN17	0.7 \pm 1.7	0.8 \pm 1.9	0.9 \pm 1.1
Lamin	0.7 \pm 1.3	0.8 \pm 1.1	0.7 \pm 1.5

^a Data representative of a typical liquid β -galactosidase assay are shown. β -Gal was assayed with *o*-nitrophenyl- β -galactosidase as described in text. The values represent means \pm SD of triplicate determination. (Reproduced from Boettner *et al.*²⁴ with permission of publisher.)

with AF-6, none of the dominant negative mutant forms show binding activity toward AF-6. This suggests that AF-6 binds Ras and Rap1 in their GTP-bound state. In addition, the data in Table I further indicate that the strength of interaction between Rap1 and AF-6 exceeds that exerted by Ras-AF-6 complexes. The findings that the affinity of interaction between Rap1 and AF-6 is greater than that between Ras and AF-6 are consistent with the kinetic studies described below.

Comparison of Interactions between AF-6, Raf, RalGDS, Phosphatidylinositol 3-Kinase δ , and Ras/Rap1, Using Histidine Prototrophy Assay and Filter Assays for β -Galactosidase Activity

We have made use of the two-hybrid system to see how AF-6 compares with c-Raf, RalGDS, and PI3K δ in terms of its ability to bind to Ras and Rap1. LBD constructs expressing AF-6-RBD1, c-RafN, RalGDS-RBD, and PI3K δ -RBD are cotransformed with LBD RasV12 and LBD Rap1E63, respectively, in the yeast strain L40 as described above. The transformants are subjected to histidine prototrophy and β -galactosidase filter assays for assessment of their respective interactions. In the first assay, the growth selection marker *HIS3* is used as a reporter, whereas in the latter transcriptional activity of the bacterial *lacZ* reporter is utilized.

To assay histidine prototrophy, pick individual transformants and spread as small patches on DO-Leu-Trp plates. After 2 days, the grown patches are replica plated first onto one or two DO-Leu-Trp plates to preclean the excess of yeast material and subsequently onto plates that in addition to leucine and tryptophan, also lack histidine (DO-Leu-Trp-His). Precleaning is important to avoid background growth.

To assay for activation of the *lacZ* reporter construct, the grown yeast patches (see above) are replica plated onto a Whatman (Clifton, NJ) No. 50 filter paper placed onto a DO-Leu-Trp plate and grown overnight. The filter with the yeast is placed in a container with liquid nitrogen for about 30 sec to lyse the cells, and then transferred (yeast cells facing up) in a petri dish containing a Whatman filter No. 3 presoaked in Z buffer/X-Gal solution (~2.5 ml of Z buffer/X-Gal solution per petri dish). Incubate at 30° and check periodically for the appearance of blue colonies.

As shown in Fig. 2, RasV12 is able to interact with all targets, the strongest interaction being with c-Raf. Rap1E63 shows strong interaction with AF-6 and RafN, but no association is observed with PI3K δ and only weak to no interaction is observed with c-Raf. This indicates that both GTPases, Ras and Rap1, use effector molecules that are only partially identical and that they exhibit differential binding profiles toward the targets listed above.

Thermodynamic and Kinetic Characterization of Interaction between Ras/Rap1 and Ras-Binding Domain of AF-6

To study protein interactions and their biochemical characterization, large quantities of protein (in the range of milligrams) are required. Al-

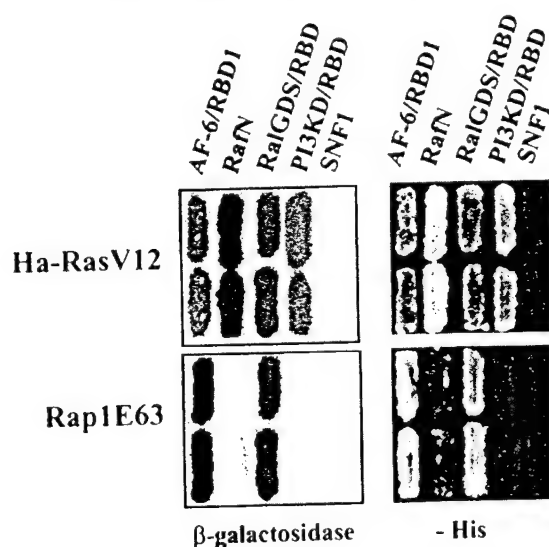


FIG. 2. Analysis of interactions between Ras/Rap1 and their effectors, using histidine prototrophy and filter assay for β -galactosidase. The LexA two-hybrid tester strain, L40, was transformed with plasmids expressing Ras and Rap1 mutants fused to LBD, and effectors of Ras and Rap1 fused to GAD. Transformants were assayed for β -galactosidase expression (*left*) and for their ability to grow on His⁺ plates. (Reproduced from Boettner *et al.*²⁴ with permission of publisher.)

though high yields of Ras proteins can be obtained with prokaryotic expression systems, this is usually not the case for their full-length effector proteins. Therefore, most biochemical studies are restricted to protein fragments that are soluble and can be prepared in large amounts. The Ras-binding domain (RBD) has been identified for many effectors such as Raf kinase, RalGDS, PI3K, and AF-6. The RBDs of the first three effectors are similar in size, comprising 80–90 amino acids. Despite the lack of sequence homology their three-dimensional structures are highly related, namely, they all show the ubiquitin fold.^{13–15} In contrast, the RBD1 of AF-6 appears to be larger. The above-described two-hybrid studies, as well as biochemical studies,¹⁶ indicated that the N-terminal part comprising the first 141 amino acids is a stable domain competent for strong binding to Ras and Rap1. This fragment was used for thermodynamic and kinetic investigations.

Methods to Quantitate Ras/Rap1 and AF-6 Interactions

Fluorescence Titration

Fluorescence is a property of many biological macromolecules that is widely used in interaction studies.¹⁷ Two different approaches allowing fluorescence measurements are commonly employed. Either intrinsic fluorescence contributed by, for example, tryptophan residues in the protein, or extrinsic fluorescence, using chemically attached fluorescent label, may be used for monitoring binding to ligands or other proteins. Although Ras does not contain tryptophan residues, the mutant Y32W, located in switch I (effector region), shows a small decrease in fluorescence when the bound GTP is hydrolyzed to GDP.¹⁸ Small changes in fluorescence intensity of RasY32W are observed with RalGDS–RBD (decreased fluorescence¹⁹) and Raf–RBD (increased fluorescence²⁰). This effect is larger at lower temperatures and can in principle be used for titration experiments. However, these experiments are feasible only for RalGDS–RBD, because it contains no tryptophan residues. The high background fluorescence caused

¹³ N. Nassar, G. Horn, C. Herrmann, A. Scherer, F. McCormick, and A. Wittinghofer, *Nature (London)* **375**, 554 (1995).

¹⁴ L. Huang, F. Hofer, G. S. Martin, and S.-H. Kim, *Nat. Struct. Biol.* **5**, 422 (1998).

¹⁵ E. H. Walker, O. Perisic, C. Ried, L. Stephens, and R. L. Williams, *Nature (London)* **402**, 313 (1999).

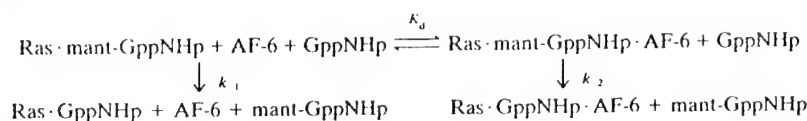
¹⁶ T. Linnemann, M. Geyer, B. K. Jaitner, C. Block, H. R. Kalbitzer, A. Wittinghofer, and C. Herrmann, *J. Biol. Chem.* **274**, 13556 (1999).

¹⁷ L. Brand and M. L. Johnson (eds.), *Methods Enzymol.* **278** (1997).

¹⁸ K. Yamasaki, M. Shirouzu, Y. Muto, J. Fujita-Yoshigaki, H. Koide, Y. Ito, G. Kawai, S. Hattori, S. Yokoyama, S. Nishimura, and T. Miyazawa, *Biochemistry* **33**, 65 (1994).

¹⁹ C. Herrmann, G. Horn, M. Spaargaren, and A. Wittinghofer, *J. Biol. Chem.* **271**, 6794 (1996).

²⁰ J. R. Sydor, M. Engelhardt, A. Wittinghofer, R. S. Goody, and C. Herrmann, *Biochemistry* **37**, 14292 (1998).



SCHEME 1.

by the tryptophan residues present in Raf-RBD (one) and in AF-6-RBD1 (two) make a direct titration impossible. Nonetheless, as we discuss below, kinetic measurements using this RasY32W mutant, together with the stopped-flow technique, allow a detailed analysis of Ras/AF-6-RBD1 complex formation.

Another method to characterize the biochemistry of Ras-like GTPases makes use of the fluorescent 2',3'-*N*-methylantraniloyl (mant) group attached to the ribose moiety of the nucleotide bound by the GTPases.^{21,22} Binding of mant-GDP or mant-GTP to Ras or Rap1 results in a more than 2-fold increase in fluorescence. A small decrease in fluorescence intensity is observed when Raf-RBD or AF-6-RBD1 is bound.²⁰ As seen with the Y32W mutant, this effect diminishes with increasing temperature. However, in this case, a direct titration of Ras/Rap1-mant-GppNHp with AF-6-RBD1 is feasible with a highly accurate fluorescence detection system.¹⁶ A broader application of the mant label is described in the following sections.

Inhibition of Guanine Nucleotide Dissociation: GDI Assay

As mentioned above, the dissociation of mant-nucleotides from Ras-like GTPases results in a large decrease in fluorescence intensity. It has been further observed that binding of effectors to the Ras proteins inhibits the dissociation of the bound nucleotide.²³ These features have resulted in the development of a method that allows quantification of all Ras GTPase-effector interactions. To block the GTPase activity of Ras or Rap1, the following experiments all use the nonhydrolyzable GTP analog GppNHp (guanylyl-5'-yl imidodiphosphate). For example, the Ras-AF-6 interaction is described below. When Ras-mant-GppNHp is incubated together with a large excess of nonlabeled GppNHp, the latter quantitatively displaces the mant-nucleotide (Scheme 1). Because of the large excess of GppNHp, virtually no rebinding of mant-GppNHp occurs, and thus this part of the reaction is ignored in Scheme 1. Equation (1) describes the dependence of the observed dissociation rate constant of mant-GppNHp (k_{obs}) on the AF-6 concentration. The rapid equilibration of the Ras-AF-6 complex is

²¹ H. Rensland, A. Lautwein, A. Wittinghofer, and R. S. Goody, *Biochemistry* **30**, 11181 (1991).

²² C. Lenzen, R. H. Cool, and A. Wittinghofer, *Methods Enzymol.* **225**, 95 (1995).

²³ C. Herrmann, G. A. Martin, and A. Wittinghofer, *J. Biol. Chem.* **270**, 2901 (1995).

illustrated by kinetic studies (see below) and is a prerequisite for Eq. (1) to hold. The rate of nucleotide dissociation is measured in the presence of different AF-6 concentrations, and this essentially corresponds to a titration experiment with k_{obs} as a readout. The K_d , describing the affinity of AF-6 for Ras, is then retrieved by fitting the values of k_{obs} obtained at different AF-6 concentrations to Eq. (1).

$$k_{\text{obs}} = k_{-1} - (k_{-1} - k_{-2}) \{ (\text{Ras}_0 + \text{AF-6}_0 + K_d) - [(\text{Ras}_0 + \text{AF-6}_0 + K_d)^2 - 4\text{Ras}_0\text{AF-6}_0]^{1/2} \} / (2\text{Ras}_0) \quad (1)$$

where Ras_0 and AF-6_0 denote total concentrations of the Ras protein and AF-6-RBD1, respectively, and K_d is the equilibrium dissociation constant of the complex.

Nucleotide Loading. The synthesis of the fluorescent nonhydrolyzable GTP analog, mant-GppNHp, has been described.²² To exchange the GTPase-bound GDP for the nonhydrolyzable GTP analog, the Ras protein (20 mg/ml) is incubated for 1 hr at 20° with alkaline phosphatase (2 U/mg) and a 2-fold excess of mant-GppNHp in the presence of 200 mM ammonium sulfate. The alkaline phosphatase hydrolyzes GDP, thereby quantitatively loading mant-GppNHp (or GppNHp) onto Ras. In the case of Rap1, 10 mM EDTA is also included. Furthermore, excess nucleotide and salt are removed by gel filtration and the mant-GppNHp-bound Ras protein is thereby transferred into the desired buffer.

GDI Assay. The GDI assay is performed by thermostating 50 nM Ras·mant-GppNHp and varying concentrations of AF-6-RBD1. This is done in fluorescence cuvettes at 37°. The buffer contains 5 mM MgCl_2 , 20 mM Tris (pH 7.5), and NaCl, to set the desired ionic strength. To obtain reliable results, 8 to 12 different AF-6-RBD1 concentrations, ranging below, near, and up to 5 times the K_d value should be tested. The dissociation (displacement) of the mant-nucleotide is initiated by the addition of 100 μM GppNHp, and the fluorescence is excited at 360 nm and monitored at 450 nm. An exponential decay curve is fitted to the fluorescence time trace, yielding k_{obs} . These data are plotted versus the effector concentration, and the K_d value is obtained from the fit according to Eq. (1).

The GDI assay has been applied to many different Ras proteins, their mutant variants, and different effectors such as Raf, RalGDS, and AF-6.^{16,19,23} For AF-6-RBD1, a K_d value of 0.25 μM was reported for Rap1A, which binds 12 times more strongly than Ras (K_d of 3 μM ¹⁶). These data are consistent with the two-hybrid results described above.

Stopped-Flow Technique

The dynamics of Ras/Rap1 and AF-6 interactions have been investigated by means of stopped flow. In this technique, two solutions containing

Rap1·mant-GppNHp and AF-6-RBD1, for example, are rapidly mixed (within 1 msec) and the time trace of the fluorescence change due to association of the proteins is recorded. In contrast to equilibrium fluorescence titration, the stopped-flow technique can be applied even when small changes in fluorescence intensity occur (<5%). In a stopped-flow experiment, this small fluorescence change is sufficient for monitoring the binding, whereas in titration experiments, it is difficult to take many readings for the accurate determination of the K_d value. Stopped-flow experiments yield dissociation and association rate constants (k_{off} and k_{on}) and from their ratio the K_d value can be calculated.

As in the titration experiment, the fluorescence is excited at 360 nm. However, all light emitted above 400 nm is detected with the use of a cutoff filter. The concentration of AF-6-RBD1 should be at least 5-fold in excess of Rap1·mant-GppNHp, to fulfill pseudo first-order conditions and to allow a single-exponential fit to the recorded trace. From this fit, the obtained k_{obs} is plotted versus the concentration of AF-6-RBD1. According to Eq. (2), the slope of the fitted straight line corresponds to k_{on} and the intercept yields k_{off} .

$$k_{obs} = k_{on}[AF-6-RBD1] + k_{off} \quad (2)$$

In many cases, as with Rap1/AF-6-RBD1, k_{off} is small and therefore not reliably obtained by this extrapolation. An accurate k_{off} value can, however, be obtained by a displacement experiment. In this case, the Rap1·mant-GppNHp·AF-6-RBD1 complex is placed in one syringe of the stopped-flow apparatus and is mixed with a large excess (>20-fold) of nonlabeled Rap1·GppNHp from another syringe. Like in the GDI assay, the time trace is fitted by an exponential curve, in this case yielding k_{off} , the dissociation rate constant of AF-6-RBD1 and Rap1. This method has been used to characterize the Ras and Rap1 binding kinetics with AF-6-RBD1,¹⁶ which are included in Table II. A more than 10% change in fluorescence obtained with saturating amounts of AF6-RBD1 allows comfortable detection of the fluorescence transience.

As a complement to the use of the mant-labeled fluorophore, we also used the intrinsic fluorescence of RasY32W in our stopped-flow assay. The experiments are carried out according to the evaluation strategy described above; however, in this case the fluorescence excitation is set at 290 nm and detection is through a 320-nm cutoff filter. In Fig. 3A, a typical fluorescence trace is shown, demonstrating a small fluorescence change. This is mainly because RasY32W is not sensitive to effector binding,²⁰ and because of the large background of AF-6-RBD1, owing to the presence of two tryptophans. To reduce the background, we use a concentration of AF-6-RBD1 no more than 5-fold in excess of RasY32W·GppNHp. Furthermore, we have chosen a low temperature, at which the change in fluores-

TABLE II
 RESULTS OBTAINED BY STOPPED-FLOW EXPERIMENTS^a

Complex	$k_{on}(\mu M^{-1} \text{ sec}^{-1})$	$k_{off}(\text{sec}^{-1})^b$	$k_{off}(\text{sec}^{-1})^c$	$K_d(\mu M)^d$
RasY32W·GppNHp ^e	19	13	11	0.58
Ras·mant-GppNHp ^f	6.4	20.8	15.3	2.4
Rap1·mant-GppNHp ^f	11.9	—	2.6	0.22

^a T. Linnemann, M. Geyer, B. K. Jaitner, C. Block, H. R. Kalbitzer, A. Wittinghofer, and C. Herrmann, *J. Biol. Chem.* **274**, 13556 (1999).

^b Obtained from the intercept of the linear fit (see text).

^c Obtained from displacement experiment.

^d Calculated from k_{off}/k_{on} .

^e 20 mM Tris (pH 7.5), 5 mM MgCl₂.

^f 20 mM Tris (pH 7.5), 5 mM MgCl₂, 100 mM NaCl.

cence is larger. In addition, NaCl is avoided in the buffer in order to have tighter binding. The observed rate constants are plotted in Fig. 4 versus the concentration of AF-6-RBD1, and the slope of the fitted straight line yields $k_{on} = 19 \mu M^{-1} \text{ sec}^{-1}$. However, the intercept corresponding to $k_{off} = 13 \text{ sec}^{-1}$ has an uncertainty of at least 50%. Therefore, we also performed displacement experiments, such as the typical trace shown in Fig. 3B. These experiments result in a much more reliable value of $k_{off} = 11 \text{ sec}^{-1}$.

This example emphasizes the strength of the stopped-flow technique. In addition to the kinetic constants, the K_d value can also be obtained using $K_d = k_{off}/k_{on}$ with only a 1.5% change in fluorescence intensity. This small change in fluorescence would not allow determination of the affinity by titration experiments. In Table II, the results obtained from the RasY32W mutant and mant labeling are compared. The results of the two systems agree well with each other, despite the different labels and the different salt concentrations used. Also, the K_d values obtained by stopped flow (Ras, 2.4 μM ; Rap, 0.22 μM) correlate well with the K_d values derived from the GDI assay (Ras, 3 μM ; Rap1, 0.25 μM).¹⁶ Furthermore, as observed for the interaction of Ras and the effector Raf,²⁰ the interaction of Ras with AF-6-RBD1 is highly dynamic, with the half-life of the complex being 60 msec (at 10°).

Establishment of Stable Ras- and Rap1-Expressing Cell Lines, Using Retroviral Gene Transfer to Study *in Vivo* Interactions between Ha-Ras/Rap1 and AF-6

Background and General Principles

The generation of stable transfectants has become a common practice to investigate the functions of specific GTPases. Most cell lines expressing

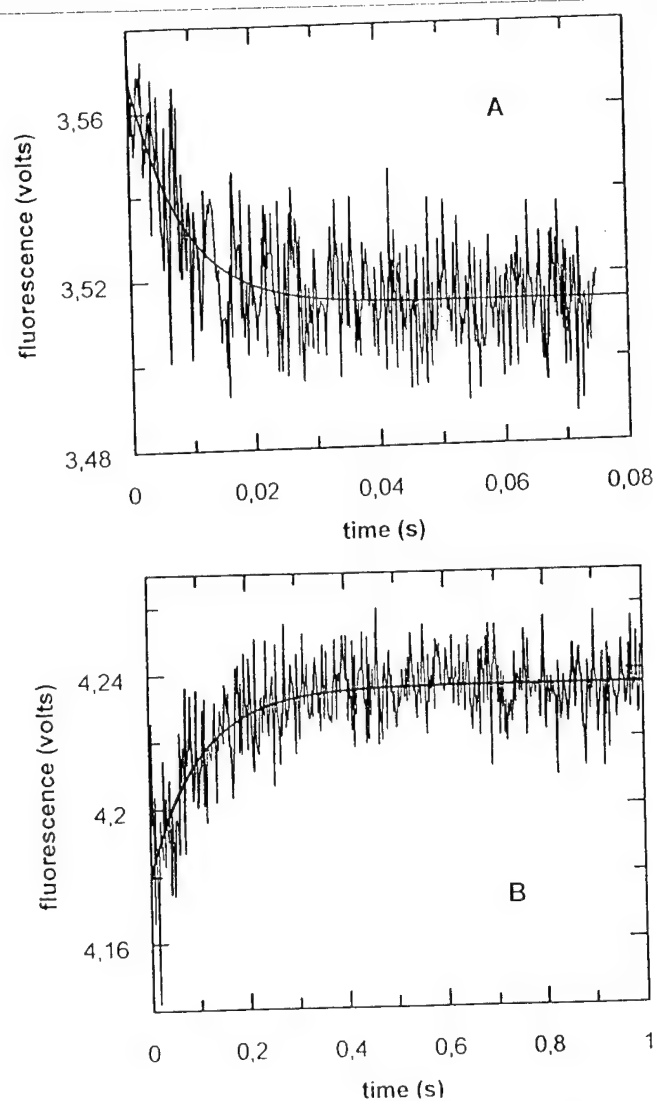


FIG. 3. Stopped-flow experiments. Fluorescence excitation was at 290 nm, and detection was through a 320 nm-cutoff filter. The buffer used consisted of 20 mM Tris (pH 7.5)–5 mM MgCl_2 , and experiments were carried out at 10°. The graphs show the experimental trace and the exponential fit. (A) Association of 1 μM RasY32W·GppNHp with 5 μM AF-6-RBD1; (B) dissociation of 2 μM RasY32W·GppNHp·AF-6-RBD1 by displacement with 40 μM Ras·GppNHp.

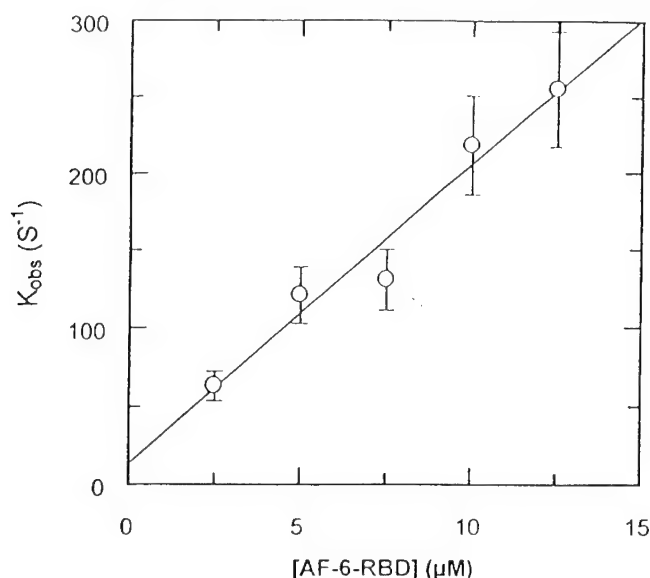


FIG. 4. Binding kinetics of RasY32W/AF-6-RBD1. k_{obs} values obtained in stopped-flow experiments, as in Fig. 3A, are plotted versus AF-6-RBD1 concentrations. According to Eq. (2), the fitted straight line yields k_{on} and k_{off} (see Table II).

dominant active or negative mutant forms of GTPases have been generated by plasmid transfection and subsequent selection for plasmid-encoded marker genes. A more refined method for the stable introduction of Ras-type cDNAs into various acceptor cell lines is viral transduction. The efficiency of gene transfer into cell lines that are relatively intransigent to conventional transfection techniques may be considerably improved by this method. A second benefit offered by this procedure is that transduced host cells tend to lose their inserted sequences to a much lesser extent than sequences that have been conventionally transfected, when kept in culture over a long period of time. The use of packaging cell lines allows us to produce viral particles that are infectious, but cannot replicate once they have entered the host cell. Retroviruses recognize specific receptors to enter their host cells. Ecotropic receptors are present on cells of mouse and rat origin. Amphotropic receptors are present on rodent cells, as well as cells of many other species, including humans. It is possible to use an ecotropic virus in nonrodent cell lines by building the ecotropic receptor into the host cell line. Retroviruses, upon invasion, reverse transcribe virion RNA to generate linear, double-stranded DNA that integrates into the host cell genome.

To address questions of localization and interaction of the AF-6 protein in cells expressing constitutively active forms of Ras and Rap1, cell lines are generated by using retroviral vectors.²⁴ LinXA cells [an amphotropic packaging line provided by G. Hannon (Cold Spring Harbor Laboratories, Cold Spring Harbor, NY)] are transfected with a pBABE²⁵ or pWZL vector²⁶ containing either the RasV12 or RapE63 insert. The virus obtained is then used to infect Madin-Darby canine kidney (MDCK) cells (nontransformed dog epithelial cells). These cell lines consist of a population of transduced cells, thus averaging out effects of retroviral integration. A detailed protocol of retroviral transfection and infection in MDCK cells is given below.

Materials

Retroviral Packaging Cell Line. LinXA (for host cell line bearing the amphotropic receptor) and LinXE (for host cell line bearing the ecotropic receptor) packaging cell lines are a gift from G. Hannon (Cold Spring Harbor Laboratories). Alternatively, the Bosc 23 packaging cell line (for host cell line bearing the ecotropic receptor) or Bing Cak 8 (for host cell line bearing the amphotropic receptor) may be obtained from the American Type Culture Collection (ATCC, Manassas, VA) with permission from Rockefeller University (New York, NY).

Media and Solutions for Retroviral Transfection and Infection. The medium used for LinX packaging cells is Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL, Gaithersburg, MD) containing 10% (v/v) fetal bovine serum (FBS; HyClone). The MDCK cells are grown in DMEM containing 10% (v/v) FBS, 1% (v/v) penicillin-streptomycin, and 20 mM HEPES (GIBCO-BRL). Solutions required are 2.5 M CaCl₂-0.01 M HEPES (pH 5.5) with NaOH (sterile filter, aliquot, and store at -20°) and 2× BBS: 50 mM N,N-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 280 mM NaCl, 1.5 mM Na₂HPO₄, pH ~7.00 (sterile filter, aliquot, and store at -20°). Make batches between 0.05 pH units below and above 7.00 and test which works the best. Polybrene (Sigma, St. Louis, MO) is used at a stock concentration of 8 mg/ml.

Retroviral Transfection and Infection of Host Cell Line

1. Plate out 6.5×10^5 LinX packaging cells per well of a six-well plate. Allow the cells to settle and begin to adhere before returning them

²⁴ B. Boettner, E. Govek, J. Gross, and L. Van Aelst, *Proc. Natl. Acad. Sci. U.S.A.*, **97**, 9064 (2000).

²⁵ J. P. Morgenstern and H. Land, *Nucleic Acids Res.* **18**, 3587 (1990).

²⁶ G. J. Hannon, P. Sun, A. Carnero, L. Y. Xie, R. Maestro, D. S. Conklin, and D. Beach, *Science* **283**, 1129 (1999).

to the incubator (approximately 10 min). Incubate overnight at 37°, 5% CO₂.

2. When the cells have reached approximately 70% confluency, change the medium gently (cells tend to lift easily) and incubate between 1 and 4 hr at 37°C, 5% CO₂.

3. Aliquot the DNA into a sterile 1.5-ml Eppendorf tube. It is best to do an initial titration using a β -galactosidase (β -Gal) construct as a readout for transfection and infection efficiency in order to determine the optimal amount of DNA for each host cell type. However, 6 μ g appears to work well in most cases. Dilute DNA to a total of 225 μ l with sterile water. To this tube add 25 μ l of 2.5 M CaCl₂-0.01 M HEPES (pH 5.5).

4. Next, bubble this mixture with a pasteur or 1-ml disposable plastic pipette, using a mechanical pipette aid. At the same time, add 250 μ l of 2 \times BBS dropwise. Add 500 μ l (total) to one well of a six-well plate dropwise around the plate. Incubate for 12-16 hr at 37°, 5% CO₂. (Two to 4% CO₂ is actually optimal at this step, but 5% works as well.)

5. Change the medium and incubate for 60 hr at 32°, 5% CO₂.

6. Remove the virus-laden medium from well and filter through a 0.45- μ m pore size syringe filter. Add to one well of host cells and subsequently add Polybrene to a final concentration of 8 μ g/ml. Host cells should be plated at a concentration that will give confluency, but not overcrowding, within 2 to 3 days.

7. Spin the cells for 1 hr at room temperature at 1700 rpm in a Beckman (Fullerton, CA) tabletop centrifuge.

8. Additional rounds of infection may give better infection efficiencies, depending on the packaging line and host cell type. Between one and six rounds may be necessary, as suggested by the ATCC, when using the Bosc cell packaging line from Rockefeller University. However, in most cases, one round appears to be sufficient with the LinX packaging cell line. Incubate the cells overnight at 32°, 5% CO₂.

9. On the next day, change the medium and incubate the cells at 37°, 5% CO₂ until the cells become confluent.

10. Transfer the cells to a 10-cm plate and incubate overnight at 37°, 5% CO₂. Add an appropriate amount of antibiotic after the transfer, but not before 48 hr after the medium was last changed. Selection may be started at the time of transfer to a large plate; however, it is best to make sure that the cell density will support viability after selection has begun. This will be dependent on host cell type and can be checked ahead of time using a β -Gal construct. Titration of appropriate antibiotics should also be done ahead of time. Generally, if puromycin is used as the selection drug, 3 days should be adequate to select out cells containing plasmid. For the generation of MDCK RasV12 and RapE63 stable cell lines, we use 10 μ g/ml for selection and 5 μ g/ml for maintenance. For the analysis of

RasV12- and RapE63-expressing clones, we have made use of anti-Ras and anti-Rap1 monoclonal antibodies obtained from Transduction Laboratories (Lexington, KY).

We have observed that expression of Rap1 does not disturb cell-cell adhesion, whereas cell-cell adhesion complexes in RasV12-expressing clones are disturbed. Furthermore, coimmunoprecipitation experiments using the above-described cell lines have been performed to show *in vivo* association between Ras/Rap1 and AF-6.²⁴

Conclusions

In this chapter, we have presented a spectrum of investigative approaches that served to demonstrate specific protein interactions between the Ras/Rap1 GTPases and their potential effector molecule AF-6 *in vivo* and *in vitro*. The two-hybrid analysis and the investigation of the kinetic and thermodynamic properties of Ras-AF-6 and Rap1-AF-6 complexes led to the same overall outcome, namely, that Rap1 appears to form the tightest complex with AF-6-RBD1. These data suggest a role for AF-6 as an effector in Rap1-mediated signaling. However, we cannot exclude its involvement in Ras-induced activities. Further investigations of the function of Ras/Rap1 and AF-6 in different biological contexts will shed more light on these interactions.

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Using cDNA-Representational Difference Analysis (cDNA-RDA) in Combination with Microarrays to Identify Rac Regulated Genes

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1. Introduction

Small GTPases of the Ras superfamily are molecular switches which cycle between an active guanosine triphosphate (GTP)-bound and an inactive guanosine diphosphate (GDP)-bound state. They integrate signals from the cell surface to the nucleus, regulating important cellular activities. For example, Ras itself is activated when extracellular growth factors such as platelet derived growth factor (PDGF) or epidermal growth factor (EGF) bind to their receptors at the cell surface. This activation of Ras ultimately leads to changes in the transcriptional activity of the cell, e.g., via the canonical mitogen activated protein kinase (MAPK) cascade. Constitutively activated, mutant forms of Ras such as RasV12 are found frequently in human tumors, and it is widely assumed that this oncogene acts via transcriptional activation of growth and proliferation pathways.

Although the Rho family members—including Rho, Rac, and Cdc42—are best known for control of the actin cytoskeleton, they have also been linked to transcriptional activation. For example, activation of Rac triggers the activation of p38 and JNK MAPKs, as well as NF κ B pathways (*1*). Furthermore, numerous studies support a role for Rac in the proliferation, invasion, and the control of cell adhesion. All these events occur over a longer time-scale compared to the short-term changes of the actin cytoskeleton, and it is often assumed that these events require transcriptional activation. However, although pathways leading from Rac to the nucleus have been identified, information about genes regulated by Rac (or other members of the Rho GTPase family) remains scarce.

Within the last decade, several methods have been developed to identify changes in gene expression. These include serial analysis of gene expression

(SAGE) (2), differential display (DD) (3,4), representational difference analysis of cDNAs (cDNA-RDA) (5,6), and suppression subtractive hybridization (SSH) (7,8). Each of these techniques has its limitations. For example, Harris et al. identified differentially expressed genes in Aflatoxin B1-treated hepatocytes, using in parallel DD, cDNA-RDA, and SSH, and each of these three methods found a small, nonoverlapping set of differentially expressed genes (9). More recently, expression analysis utilizing microarray technology has become available (10). This technique depends on the availability of reliable cDNA clones that can be arrayed, whereas the former methods allow the identification of novel sequences in incompletely characterized organisms. One major advantage of cDNA-RDA compared to other methods is its low level of false-positives, because RDA eliminates those fragments which are present in both DNA populations. Furthermore, cDNA-RDA does not require sophisticated equipment.

As an example, we describe protocols for using cDNA-RDA to identify genes differentially expressed between cell lines with or without induction of Rac1 expression (under the control of an inducible promoter). cDNA-RDA is a modified form of RDA, a polymerase chain reaction (PCR)-based differential cloning method (Fig. 1) (11,12). With this technique, one DNA population

Fig. 1. (see opposite page) Principle of cDNA-RDA. Flowchart of cDNA-RDA. (A) From cells either induced or left uninduced for expression of the gene of interest, representations are prepared by isolation of mRNAs, synthesis of cDNAs, restriction digest with *DpnII*, ligation to adaptors, and amplification by PCR. The adaptors are subsequently removed by digestion in order to use the representation as the driver in a cDNA-RDA experiment. To use the representation as the tester, the adaptors are cut off and replaced by newly ligated adaptors of different sequence. Then, the tester from one sample is hybridized to an excess of driver from the other sample and sequences enriched in the tester are selectively amplified by PCR to obtain the difference product one (DP1). Finally, the DP1 is used as the tester in a new round of hybridization and amplification to result in difference product two (DP2). Note that by performing two sets of reactions in parallel, using cells from each of the two samples once as tester and once as driver against the other sample, up- as well as downregulated genes can be identified. (B) The three possible outcomes of the tester/driver hybridization. If a sequence is unique to the tester or present at a higher molar ratio in the tester than in the driver, it will be exponentially amplified. If a sequence is found in both driver and tester to equal amounts, only the strand from the tester population bears the adaptor and the sequence will be linearly amplified. If the sequence is found only in the driver, neither strand contains the adaptor sequence and the sequence will not be amplified. (C) A typical difference product after two rounds of cDNA-RDA visualized by agarose gel electrophoresis. A difference product consists of a series of visible bands superimposed on a "smear." As indicated, each band may contain fragments of several genes whose different sizes can not be resolved on an agarose gel. Furthermore, candidate genes may be contained in the "smear." Finally, different fragments of the same gene can occur at different positions in the gel, since cDNA-RDA is based on digested cDNA.

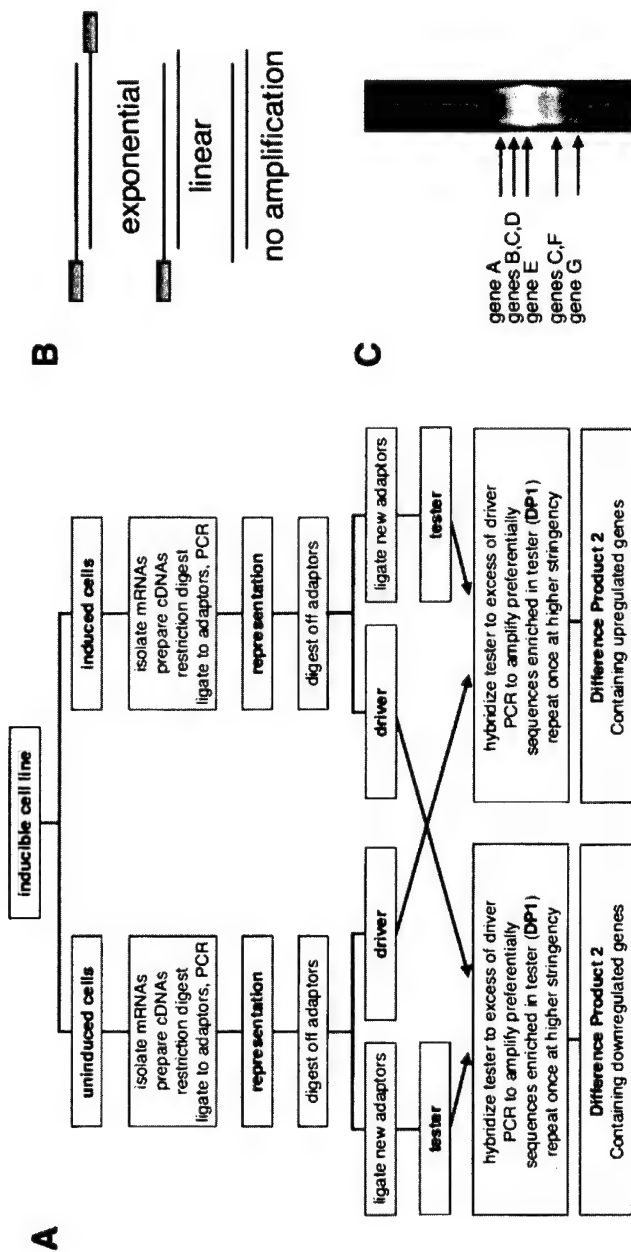


Fig. 1.

(the driver) is hybridized in excess against a second population (the tester) to remove common hybridizing sequences, thereby enriching target sequences unique to the tester. RDA relies on the use of representations of the DNAs of interest. In brief, a representation is prepared by restriction digestion of the DNA (e.g., *DpnII* in the case of cDNA-RDA), ligation of adaptor oligonucleotides, and subsequent PCR amplification.

One major task is the analysis of the difference product resulting from the cDNA-RDA experiment (**Fig. 1C**). To identify the most promising candidate genes, we and others (*13*) used the microarray technique as detailed here (**Fig. 2**). Only the clones with the highest differential expression as judged by microarray analysis were sequenced and further pursued. By doing so, we were able to significantly increase the number of clones screened per cDNA-RDA experiment.

2. Materials

2.1. Equipment

1. PCR machine capable of handling 0.5 mL polymerase chain reaction (PCR) tubes to perform cDNA-RDA (e.g., Perkin Elmer DNA Thermal Cycler 480).
2. PCR machine which can hold 96-well plates (e.g., Perkin Elmer GeneAmp 9600) or up to four 96-well plates simultaneously (e.g., MJ Research PTC-225) to process the samples for microarraying.
3. Speedvac (e.g., Savant DNA 100).
4. 8-channel pipettors such as LabSystems Finnpipette 4510000 (0.5–10 μ L) and 4510020 (5–50 μ L) and a repeat pipet (e.g., Brinkmann Eppendorf 22260006) are very helpful for working with 96-well plates.
5. 96-well PCR plates are obtained from Perkin Elmer (N801-0560), 96-well plates for dilutions of DNA samples are retrieved from Nunc, and 96-well plates for microarraying (with V-shaped bottom) are obtained from Corning-Costar. Plates are covered either with caps (during PCR, Perkin Elmer N801-0535) or sealing film (Sigma Z36, 968-3).
6. Ultraviolet (UV) crosslinker (Stratalinker 2400, Stratagene).
7. Minifold I dot-blotting apparatus (Schleicher and Schuell SRC096/0).
8. Hybond-N+ nylon membrane (Amersham Pharmacia Biotech RPN203B).
9. Microcon YM-30 ultrafiltration columns (Amicon) to purify and concentrate labeling reactions.
10. Vacuum oven.
11. Microarrayer, e.g., Cartesian PixSys 5500 (Cartesian Technologies, Irvine, CA).
12. Pins for the arrayer (Chipmaker 2, Telechem International).
13. Silanated glass slides (Corning).
14. Humidified hybridization chamber (Telechem International).
15. Scanner suitable for microarray fluorescence detection, such as GSI Lumonics ScanArray3000 or Axon GenePix4000.

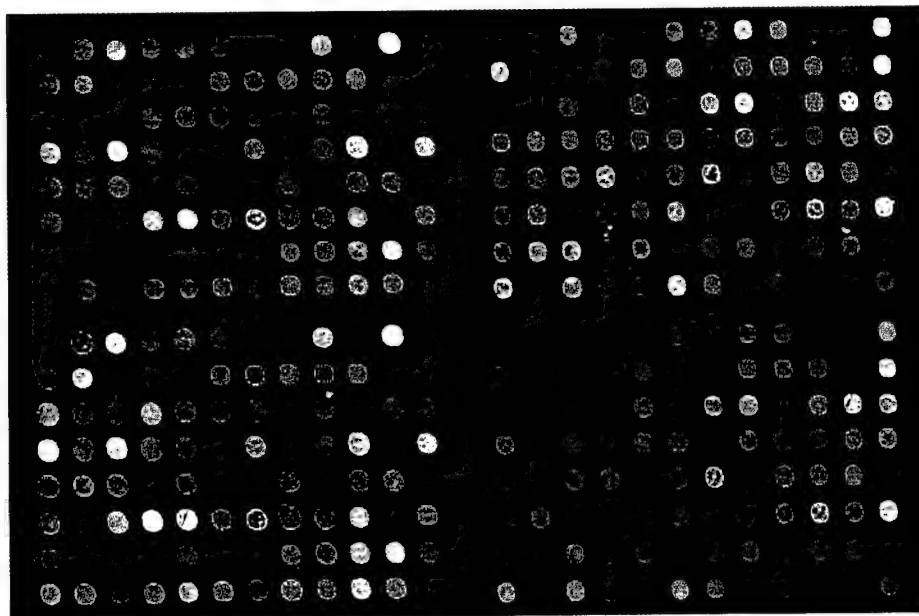


Fig. 2. Analysis of cDNA-RDA products by microarray analysis. 190 clones derived from a cDNA-RDA experiment using cells induced for RacV12 expression as the tester and uninduced cells as the driver were arrayed in duplicate. The array was then hybridized simultaneously to a representation from RacV12 induced cells (labeled in green) and to a representation from uninduced cells (labeled in red). The clones with different shades of green represent genes whose expression is upregulated as a result of RacV12 expression, whereas "yellow clones" are transcripts unaffected by RacV12 expression. Genes downregulated by RacV12 would be identified as red spots, but are absent as expected from the design of this particular experiment. Such microarraying allows rapid screening of a difference product for the clones with the highest differential expression ratio, which are then further pursued.

16. Analysis software: We made use of ScanAlyze (Stanford University) or Axon GenePix to determine features and for the quantitative analysis of the resulting TIFF files.

2.2. Enzymes and Reagents

1. Organic solvents such as 70% and 100% ethanol (EtOH), isopropanol (iPrOH), chloroform, dimethyl sulfoxide (DMSO), and phenol/chloroform/isoamylalcohol (25/24/1) saturated with TE pH 8.0 (Sigma P 2069).
2. FastTrack mRNA isolation kit (Invitrogen K1593-02).
3. CopyKit cDNA synthesis kit (Invitrogen L1311-03).
4. DpnII restriction enzyme and 10 x DpnII buffer (New England Biolabs 543L).

5. 10 mg/mL tRNA (Sigma R 8759) is used as a carrier during precipitations of small amounts of DNA.
6. Sheared salmon-sperm DNA (Stratagene 201 190, diluted to 50 ng/ μ L).
7. DNA mol-wt marker ϕ 174 *Hae*III digest (New England Biolabs 3026L).
8. T4 ligase and 10 \times T4 buffer (New England Biolabs 202S).
9. The primers listed in **Table 1**.
10. AmpliTaq polymerase, 25 mM MgCl₂, and 10 \times Taq buffer without MgCl₂ (5 U/ μ L, Perkin Elmer N8080-153).
11. Deoxynucleotide 5' triphosphate (dNTPs) (100 mM, Roche Molecular Biochemicals 1 969 064).
12. Mung bean nuclease (MBN) and 10 \times MBN buffer (New England Biolabs 250S).
13. Qiaquick gel-extraction kit (Qiagen 28704).
14. *Bam*HI restriction enzyme, 10 \times *Bam*HI buffer, and 10 mg/mL bovine serum albumin (BSA) (New England Biolabs 136S).
15. Calf intestinal phosphatase (CIP) and 10 \times CIP buffer (New England Biolabs 290S).
16. Qiaquick PCR purification kit (Qiagen 28104).
17. DNA polymerase I Klenow fragment and 10 \times reaction buffer (Amersham Pharmacia Biotech E2141).
18. Cy3-deoxycytidine 5' triphosphate (dCTP) and Cy5-dCTP for probe labeling (Amersham Pharmacia Biotech PA53021 and PA55021).
19. dRhodamine dye terminator kit for nonradioactive DNA sequencing (Perkin Elmer 403045).

2.3. Buffers

1. 3 M sodium acetate (NaAc), pH 5.2.
2. Elution buffer (EB): 10 mM Tris-HCl pH 8.5.
3. 6 \times GLB (Gel-loading buffer): 30% glycerol and 0.25% Bromophenol blue.
4. 500 mM Tris-HCl pH 8.9 (It is important to use Tris base adjusted to pH with HCl instead of Tris hydrochloride adjusted to pH with NaOH.)
5. 100 mM MgCl₂.
6. 200 mM (NH₄)₂SO₄, sterile-filtered.
7. TE buffer: 10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0.
8. 10 M ammonium acetate (NH₄Ac).
9. EE buffer: 30 mM *N*-(2-Hydroxyethyl) piperazine-*N'*-3-propane sulfonic acid (EPPS) (Sigma E 1894) pH 8.0, 3 mM EDTA pH 8.0, sterile-filtered.
10. 5 M NaCl.
11. 20 \times SCC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0.

2.4. PCR Buffers

PCR buffers are prepared from autoclaved or sterile-filtered stock solutions and autoclaved water (*see Note 1*). All PCR buffers are made immediately before use and stored on ice.

Table 1
Sequences of the Used Primers

Name	Sequence	Use	Ref.
RBgl24	AGCACTCTCCAGCCTCTCACCGCA	Representation	<i>11</i>
RBgl12	GATCTGCGGTGA	Representation	<i>11</i>
NBgl24	AGGCAACTGTGCTATCCGAGGGAA	1st round of cDNA-RDA	<i>11</i>
NBgl12	GATCTTCCCTCG	1st round of cDNA-RDA	<i>11</i>
IBgl24	TCAGCATCGAGACTGAACGCAGCA	2nd round of cDNA-RDA	<i>a</i>
IBgl12	GATCTGCTGCGT	2nd round of cDNA-RDA	<i>a</i>
SP6R	GGTGACACTATAGAATACTCAAGC	Single-colony PCR	<i>b</i>
T7E	TGTAATACGACTCACTATAGGGC	Single-colony PCR	<i>b</i>
Random nonamer	NNNNNNNNN	Probe labeling	<i>a</i>

^aRobert Lucito, unpublished results.

^bThis report.

1. PCR buffer 1 is used in **Subheading 3.2.1.3.** for the preparation of representations. This buffer consists of 67 mM Tris-HCl, pH 8.9, 4 mM MgCl₂, 10 mM 2-mercaptoethanol, 0.1 mg/mL BSA, 16 mM (NH₄)₂SO₄, 320 μM each dNTP, 1.25 μM primer RBgl24, and 0.04 U/μL (15 U/tube) Taq.
2. PCR buffer 2 is identical to buffer 1, except that the primer and the Taq polymerase are added later. This buffer is used for the first PCR in each round of cDNA-RDA described in **Subheadings 3.2.2.3.** and **3.2.3., item 4.**
3. PCR buffer 3 is also identical to buffer 1, except that primer NBgl24 (in **Subheading 3.2.2.4.**) or IBgl24 (in **Subheading 3.2.3., item 4**) are used. This buffer is employed during the second PCR in each round of cDNA-RDA.
4. PCR buffer 4 is used for the single-colony PCR described in **Subheading 3.3.3.** This buffer consists of 12% DMSO in 1 × Taq buffer supplemented with 2 mM MgCl₂, 200 μM each dNTP, 0.5 μM primer SP6R, 0.5 μM primer T7E, and 0.025 U/μL (0.625 U/well) Taq polymerase.
5. PCR buffer 5 is a standard PCR buffer used in **Subheading 3.3.5.** for reamplification. Buffer 5 is identical to buffer 4, except that dimethyl sulfoxide (DMSO) is omitted.

3. Methods

A detailed description of the various steps in cDNA-RDA and the analysis of the difference product by microarray analysis is given. More common techniques used throughout our study, such as Western, Northern, and Southern blot analysis, are not described here in detail.

3.1. Preparation of cDNAs

In order to obtain meaningful results by cDNA-RDA, the samples used for the preparation of tester and driver must be selected carefully. It is best to

differ in only one parameter and to compare two samples matched as close as possible. For this reason, we made use of an inducible system for the expression of the target gene (14–16).

1. Perform time-courses and titrate the concentration of inducer to establish optimal induction conditions for the selected inducible clones.
2. Grow approx 5×10^7 cells of one selected clone in one incubator. Split cells into two halves and induce one-half of the population. For example, in the case of MDCK cells with expression of RacV12 under the control of the tetracycline-off system, 2 d in the absence of doxycycline were sufficient to induce expression of RacV12 to the level of endogenous Rac (17). Continue to use the same batch of medium for tester and driver populations. Harvest approx 1×10^8 each of uninduced and induced cells by scraping into ice-cold PBS. Process immediately, or store the cell pellet at -80°C . Before continuing, check by Northern or Western blot analysis to see whether induction was successful.
3. Isolate mRNA from cells induced and not induced for expression of the gene of interest. Several commercial kits are available. We used the Fast Track kit for mRNA isolation and the CopyKit for cDNA preparation (both from Invitrogen). A yield of approx $3 \mu\text{g}$ mRNA/ 10^7 cells can be expected, depending on the cell type. Use $5 \mu\text{g}$ mRNA to generate approx $7 \mu\text{g}$ cDNA (see Note 2).

3.2. cDNA-RDA

3.2.1. Preparation of Representations

Representations are obtained by digestion of the cDNA sample of interest with a frequently cutting restriction enzyme such as *DpnII* (GATC), followed by ligation to short-adaptor oligonucleotides and amplification by PCR, using the same sequences as primers. Representations to be compared to each other by cDNA-RDA must be prepared simultaneously.

3.2.1.1. DIGESTIONS OF THE cDNAs

1. Digest the two cDNA samples derived from the induced and the uninduced cells in parallel. Each digest consists of $1.2 \mu\text{g}$ of cDNA in $10 \mu\text{L}$ $10 \times$ *DpnII* buffer, $10 \mu\text{L}$ $10 \text{ U}/\mu\text{L}$ *DpnII*, and H_2O to a total vol of $100 \mu\text{L}$ overnight (16 h) at 37°C (see Note 3).
2. Add $1 \mu\text{L}$ $10 \mu\text{g}/\mu\text{L}$ tRNA, vortex, and extract twice with $100 \mu\text{L}$ phenol/chloroform. Add $10 \mu\text{L}$ 3 M NaAc pH 5.2 and $330 \mu\text{L}$ 100% EtOH (kept at -20°C), vortex, and incubate at -70°C for 10 min.
3. Spin 10 min at $15,000g$ at 4°C , and wash the pellets with $500 \mu\text{L}$ 70% EtOH (kept at -20°C). Spin again and remove wash solutions as completely as possible by pipetting. Dry in a speedvac at medium heat and resuspend carefully in $12 \mu\text{L}$ EB (see Note 4).

4. Transfer 2 μL to a tube containing 8 μL H_2O and 2 μL 6 \times GLB. Load the samples onto a 2% agarose gel, side-to-side with 200 ng undigested cDNA (to check digest), a dilution series of 50–100–200 ng sheared salmon-sperm DNA (to check recovery and concentration), and 200 ng of $\phi 174$ *Hae*III as the marker.

3.2.1.2. LIGATION

The ligation is preceded by an incubation step ensuring optimal conditions for the adaptors to hybridize to the cDNA fragments as follows:

1. To the remaining 10 μL of each cDNA digest, add 7.5 μL 62 μM RBgl24, 7.5 μL 62 μM RBgl12, 3 μL of 10 \times T4 buffer, and 2 μL H_2O .
2. Mix well and transfer to a heatblock (holes filled with glycerol for optimal thermal contact) at 55°C.
3. Transfer the block with the two tubes to the cold room, and allow the temperature to decrease to 10–15°C over the next 90 min (check).
4. Add 1 μL T4 ligase to each tube, mix by pipetting, and incubate for 16 h at 12°C.

3.2.1.3. AMPLIFICATION BY PCR

To ensure equal conditions during preparation of representations, these PCRs must be performed in parallel in the same PCR machine using the same master mix for both sets of reactions.

1. Add 970 μL of TE supplemented with 200 $\mu\text{g}/\text{mL}$ (20 μL of 10 $\mu\text{g}/\mu\text{L}$) tRNA to each of the two ligations, vortex, and store on ice.
2. Label 2 \times ten 0.5 mL PCR tubes, a set each for the induced and the uninduced sample, respectively. Prepare 8 mL of PCR buffer 1 and aliquot 360 μL into each of the 20 PCR tubes. Add 40 μL from each of the two diluted ligations to the ten corresponding tubes. Vortex each tube briefly before adding one drop of mineral oil to each tube.
3. Incubate tubes at 72°C for 5 min prior to cycling, to allow extension of adaptor ends. Cycle all 20 tubes together 20 \times in a two-temperature PCR cycle (1 min at 95°C and 3 min at 72°C). Finish the reaction by a final extension at 72°C for 10 min.
4. To check the outcome of the PCR, run 8 μL of the reaction from each tube + 1.6 μL 6 \times GLB on a 2% agarose gel, using $\phi 174$ *Hae*III as the marker and 200–300–400 ng sheared salmon-sperm DNA to check yield (approx 300 ng per lane can be expected). All ten aliquots prepared from the induced sample should appear identical. Also, all ten aliquots from the uninduced sample should look identical among themselves. Often, a band pattern specific for each of the two samples is observed.

5. Withdraw the PCR reactions under the mineral oil and collect each of the two samples (in total 4 mL from the ten matching tubes) in a 15-mL Falcon tube. Extract both pooled samples twice with 3 mL phenol/chloroform. Add 400 μ L 3 M NaAc and 8 mL iPrOH. Incubate for 15 min at 4°C (or overnight at -20°C), and spin at 15,000g 30 min at 4°C.
6. Wash pellets once with 5 mL 70% EtOH (kept at -20°C), recentrifuge for 5 min, remove supernatants, and dry pellets in a desiccator. Redissolve pellets in 500 μ L TE. Check recovery by diluting 2 μ L sample 1:10 with TE and running 2, 5, and 10 μ L on a 2% agarose gel with 100-250-500 ng salmon-sperm DNA as standards.
7. Estimate the amount of DNA on the gel and use it to calculate the total amount of representation recovered. Approximately 150 μ g for each of the two representations prepared can be expected. This corresponds to approx a 400-fold amplification, since the representations were made from 0.4 μ g template each (*see Note 5*).

3.2.1.4. REMOVAL OF THE ADAPTORS BY DIGESTION

Before the representations can be used in a cDNA-RDA reaction, the adaptors at their ends must be cleaved by restriction enzyme digestion.

1. Use 100 μ g of each of the two representations prepared in **Subheading 3.2.1.3.** for a digest to remove adaptors. In a 2-mL Eppendorf tube, add 100 μ g representation to a reaction mix consisting of 100 μ L 10 \times DpnII buffer, 100 μ L 10 U/ μ L DpnII, and water to 1 mL final vol. Incubate overnight at 37°C, preferentially in an incubator.
2. Extract twice with 1 mL phenol/chloroform. Add 100 μ L 3 M NaAc and 1 mL iPrOH. Incubate for 15 min at 4°C (or overnight at -20°C), and spin the two tubes at 15,000g at 4°C for 15 min. Wash pellets once with 1 mL 70% EtOH (kept at -20°C), and carefully dry pellets in a speedvac. Redissolve carefully in 100 μ L TE by pipetting and vortexing.
3. Check recovery and completeness of digestion by preparing 10 μ L of a 1:10 dilution and run 1-, 2.5-, and 5- μ L samples on a 2% agarose gel. Include 200 ng samples of the undigested representations (*see Subheading 3.2.1.3.*). The band pattern before and after digestion should be identical, except that after digestion it will be shifted slightly downward because of the removal of 48 bp of adaptor sequence. Use 100-250-500 ng salmon-sperm DNA to check yield. Adjust concentrations to 500 ng/ μ L with TE, pipet, and revortex.

3.2.2. First Round of cDNA-RDA

Since cDNA-RDA identifies only samples upregulated in one sample relative to the other, we perform two cDNA-RDA experiments in parallel (*see Fig. 1*). To identify downregulated genes, use the representation derived from induced cells as the driver and the tester derived from uninduced cells. To identify upregulated genes, use the representation derived from uninduced cells as the driver, and

the tester derived from induced cells. In this experiment, it is advisable to add the induced gene of interest back to the driver, since this sequence may otherwise constitute a major part of the difference product (*see Note 6*).

3.2.2.1. LIGATION OF NEW ADAPTORS TO TESTER

1. Set up in parallel two ligations to obtain testers from each of the two samples. Each ligation contains 2 μL of 500 ng/ μL digested representation (*see Subheading 3.2.1.4.*), 7.5 μL 62 μM NBgl24, 7.5 μL 62 μM NBgl12, 3 μL 10 \times T4 buffer, and 10 μL H_2O .
2. Mix well and transfer to a heatblock (holes filled with glycerol for optimal thermal contact) at 55°C. Transfer the block with the tubes to the cold room and allow the temperature to fall to 10–15°C during the next 90 min.
3. Add 1 μL T4 ligase, mix by pipetting, and incubate for 16 h at 12°C.
4. Add 70 μL TE supplemented with 20 $\mu\text{g}/\text{mL}$ tRNA, and store on ice.

3.2.2.2. HYBRIDIZATION

1. Adjust one thermoblock with glycerol- or mineral oil-filled holes to 67°C and another one to 98°C.
2. For each of the two reactions, add 80 μL of 500 ng/ μL (40 μg) digested representation, corresponding to the driver (*see Subheading 3.2.1.4.*), to 40 μL of 10 ng/ μL (0.4 μg) representation ligated to new adaptors (*see Subheading 3.2.2.1.*; the tester). Vortex the Eppendorf tubes and extract once with 120 μL phenol/chloroform. Add 30 μL 10 M NH_4Ac , vortex, and add 380 μL EtOH (kept at –20°C). Incubate at –70°C for 10 min, warm the samples for 2 min at 37°C, and spin at 4°C at 15,000g for 20 min. Wash twice with 500 μL 70% EtOH, and dry carefully in a speedvac (up to 2 min, without heating).
3. Resuspend each pellet in 5 μL EE (*see Subheading 2.3., item 9*) by pipetting and vortexing 4 \times for 30 s each. Spin down very briefly and carefully add 35 μL mineral oil. Denature DNA at 98°C for 4 min, add 1.5 μL 5 M NaCl to the aqueous phase by pricking through the mineral oil with the pipet tip, and incubate at 67°C for 20 h.

3.2.2.3. FIRST PCR

1. Remove mineral oil from the hybridizations (*see Subheading 3.2.2.2.*). Add tRNA (8 μL 5 $\mu\text{g}/\mu\text{L}$) to the hybridization mix. Mix by pipetting, add 390 μL TE, and vortex.
2. Prepare 1.5 mL of PCR buffer 2, distribute 352 μL each into four PCR tubes. Add 40 μL (4 μg total DNA) from the one hybridization to two of the four tubes and 40 μL from the other hybridization to the remaining two tubes, and place them in a PCR machine kept at 72°C.
3. Add 3 μL 5 U/ μL Taq polymerase to each of the four tubes, vortex, and incubate for 5 min at 72°C. During this time, the 3' ends of the adaptor sequences will be filled in. Add 10 μL of 62 μM primer NBgl24 to each tube, vortex, add mineral

oil, and cycle 10 times in a two-temperature PCR (keep at 95°C for 1 min and at 72°C for 3 min).

3.2.2.4. MBN DIGEST AND SECOND PCR

The PCR step described in **Subheading 3.2.2.3.** selectively amplifies the sequences enriched in the tester. To improve amplification of tester-specific sequences during the second PCR amplification, the single-stranded driver DNA is digested by MBN treatment.

1. Place a water bath at 30°C and a heatblock at 98°C.
2. Pool the identical samples obtained after the first PCR (*see Subheading 3.2.2.3.*). Extract once with 600 μ L phenol/chloroform and once with 600 μ L chloroform. In a 2-mL Eppendorf tube, add 80 μ L 3 M NaAc and 1 mL isopropanol (iPrOH).
3. Incubate for 1 h at -20°C, spin 15 min at 15,000g at 4°C. Wash pellets once with 500 μ L 70% EtOH (kept at -20°C), recentrifuge for 5 min, and remove supernatants.
4. Dry pellets in a speedvac and redissolve carefully in 40 μ L EB by pipetting and vortexing. Prepare 50 μ L 2 \times MBN buffer.
5. Incubate 20 μ L from each of the two samples in parallel with 20 μ L 2 \times MBN buffer and 2 μ L 10 U/ μ L MBN enzyme for 30 min at 30°C.
6. Neutralize with 160 μ L 50 mM Tris, pH 8.9 and inactivate the MBN for 5 min at 98°C, then store on ice.
7. Prepare 1.5 mL of PCR buffer 3, distribute 360 μ L into four PCR tubes. Add 40 μ L from each sample from **step 6** to two PCR tubes. Do not combine the samples at this step. Vortex, add mineral oil, and perform 20 PCR cycles of 1 min at 95°C and 3 min at 72°C.
8. To evaluate the outcome of the PCR, run 10 μ L of the reaction from each tube + 2 μ L 6 \times GLB on a 2% agarose gel and use 100–200–300 ng sheared salmon-sperm DNA to check yield (a yield of approx 200 ng/10 μ L can be expected). In the case of a low yield, supplement each tube with 3 μ L of fresh Taq polymerase and perform three additional PCR cycles.
9. Pool the identical samples and extract them once with 600 μ L phenol/chloroform and once with 600 μ L chloroform. In a 2-mL Eppendorf tube, add 80 μ L 3 M NaAc and 1 mL iPrOH. Incubate overnight at -20°C.
10. Centrifuge for 15 min at 15,000g at 4°C. Wash pellet once with 500 μ L 70% EtOH (kept at -20°C), and dry the pellets in a speedvac.
11. Redissolve the pellets carefully in 100 μ L TE by pipetting and vortexing. Prepare 20 μ L of a 1:5 dilution and run 2.5, 5, and 10 μ L on a 2% agarose gel. Include lanes with 100–200–300–400 ng salmon-sperm DNA to check yield (approx 20 μ g/sample is to be expected). Adjust the concentration to 100 ng/ μ L and store the difference product 1 (DP1) at -20°C.

3.2.3. Second Round of cDNA-RDA

The second round of cDNA-RDA is basically a repetition of the first round, incorporating the following changes: The product of the first round of cDNA-RDA (DP1) is used as the tester, and the driver is still the digested representation. The driver to tester ratio is increased from 100:1 to 800:1. Also, a new set of primers is used.

1. Remove the NbgI24 adaptors from 5 µg of DP1 with *DpnII* following the protocol provided in **Subheading 3.2.1.4**. Resuspend after precipitation in 50 µL TE. Check the yield by agarose gel electrophoresis and adjust the concentration to 20 ng/µL with TE.
2. Ligate new adaptors to 100 ng (5 µL) of digested DP1, using the same conditions and concentrations of reagents as described in **Subheading 3.2.2.1**, but using IBgl24 and IBgl12 instead of NbgI24 and NbgI12. Incubate overnight, add 50 µL TE supplemented with 20 µg/mL tRNA, and store on ice.
3. Mix 40 µL (50 ng) of tester just ligated to new IBgl adaptors and 80 µL of 500 ng/µL (40 µg) driver (prepared in **Subheading 3.2.1.4**). Hybridize, following the instructions given in **Subheading 3.2.2.2**.
4. Perform first PCR, MBN digest, and second PCR as outlined in **Subheading 3.2.2.3** and **Subheading 3.2.2.4**, substituting primer IBgl24 for NbgI24. Adjust the concentration to 100 ng/µL and store the difference product 2 (DP2) at -20°C (see **Note 7**).

3.3. Analysis of the Obtained Difference Products

3.3.1. Southern Blot Analysis

After completion of the cDNA-RDA experiment (which will take approx 2–3 wk), the difference products (DP2) are analyzed. A first standard control is performed by Southern blot analysis, using the induced gene (e.g., *Rac*) as a positive control to probe equal amounts of each driver and tester representation, DP1, and DP2 transferred to a nylon membrane. If looking for genes downregulated by *Rac*, the hybridization signal from the *Rac* probe should be stronger in the driver than in the tester and absent from both DP1 and DP2. If looking for genes upregulated by *Rac*, the hybridization signal should be stronger in the tester than in the driver and stronger in both DP1 and DP2 are compared to tester. This signal is likely to be detected, although to a lesser extent, even when the induced gene was added back to the driver (see **Note 6**).

If the blot indicates that the cDNA-RDA experiment was successful, the individual clones contained in the DP2 are analyzed. The DP2 library is subcloned, individual inserts are amplified and revalidated by microarraying in order to sequence only the most promising candidate genes.

3.3.2. Subcloning

1. Digest 1 µg DP2 (*see Subheading 3.2.3.*) with *DpnII* as described in **Subheading 3.2.1.4.**
2. After redissolving in 20 µL EB, add 4 µL 6 × GLB and run at low voltage on a 2% agarose gel approx 4 cm distance from the wells. With a fresh scalpel, cut out the entire lane from 800–100 bp, leaving the cleaved adaptors behind.
3. Use the Qiaquick gel extraction kit to recover the DNA and ligate the adaptor-free DP2 to a bacterial expression vector (e.g., pGEM7Zf[–]) digested with *Bam*HI and dephosphorylated with CIP as described in **Subheadings 3.2.1.2.** and **3.2.2.1.** Transform into highly competent *E. coli* and plate the library.

3.3.3. Single-Colony PCR

Individual clones are directly amplified from the bacterial colonies by using the bacteria as the template in a PCR reaction with primers directed against the sequences surrounding the multiple cloning site of the vector. We suggest processing four 96-well plates from each DP2 library.

1. Prepare 2.75 mL of PCR buffer 4 (*see Note 8*). Pipet 325 µL into each well of the first column of a 96-well PCR plate. With an 8-channel pipetter, distribute 25 µL into each of the wells across the plate.
2. Pick a single white colony from the plate using a sterile pipet tip, and dip the tip into a well of the PCR plate. Repeat 94 times, inoculating each well with an individual white colony picked at random and using fresh tips. Include one well as a negative control without any template.
3. Amplify by PCR using a three-temperature program (5 min at 94°C for initial denaturation; 25 cycles of 1 min each at 94°C, 60°C, and 72°C; additional 10 min at 72°C for final elongation and hold at 4°C). Run 5 µL sample, 5 µL H₂O, and 2 µL 6 × GLB on a 2% agarose gel with ϕ 174 *Hae*III as the standard, and photograph. Store remainder of the PCR reactions at –20°C.

3.3.4. Exclusion of cDNA Clones Which Are Identical to the Induced Gene of Interest

It is to be expected that the induced gene (e.g., Rac) will be found as a difference product if the tester is derived from induced cells, even when the induced gene was added back to the driver (*see Note 6*). To prevent sequencing these clones, we identified them by dot-blotting experiments.

1. Prepare a 96-well plate with 10 µL TE in each well. Add 1 µL of each of the PCR reactions from **Subheading 3.3.3.** into the corresponding wells.
2. Following the manufacturer's instructions, place a Hybond-N+ nylon membrane in the Minifold I dot-blotting apparatus and apply vacuum. Load your DNA samples in the corresponding wells. Fix the DNA to the membrane, e.g., by UV-crosslinking.

3. Prepare 50 ng of radiolabeled double-stranded DNA probe from your induced gene (e.g., full-length *Rac1*) and hybridize to the dot-blot filter.

3.3.5. Re-Amplification for Microarraying

The amplified clones must be re-amplified in order to obtain sufficient amounts for microarraying.

1. Prepare 10 mL of PCR buffer 5. Add 100 μ L each to the wells of a 96-well PCR plate. With an 8-channel pipetter, add 1 μ L each from the PCR reactions (*see Subheading 3.3.3.*) into the corresponding well. Amplify by PCR using the following program: 1 min at 94°C for initial denaturation; 25 cycles of 1 min each at 94°C, 60°C, and 72°C; additional 10 min at 72°C for final elongation; hold at 4°C).
2. Run 5 μ L of each of the PCR products and 1 μ L 6 \times GLB on a 2% agarose gel with ϕ 174 *HaeIII* as the standard, and photograph. Transfer the samples to a Corning 96-well plate, supplement with 10 μ L 3 M NaAc and 200 μ L EtOH, and keep for 16 h at -20°C.
3. Centrifuge for 20 min at + 4°C and 2800g. Aspirate supernatants, wash with 100 μ L 70% EtOH, spin for 5 min, and aspirate supernatants. Dry the pellets in a vacuum oven at maximal 50°C, resuspend in 15 μ L of 3 \times SSC per well, and store at -20°C.

3.3.6. Microarraying

In our microarraying experiments, the clones derived from a cDNA-RDA experiment are arrayed. Representations from each driver and tester are labeled with a fluorophore, mixed, and then hybridized simultaneously to the arrayed candidate genes.

3.3.6.1. ARRAY PREPARATION

1. Array the re-amplified inserts from **Subheading 3.3.5.** on commercially prepared silanated slides, using a Cartesian PixSys 5500 or equivalent.
2. Place the array in a humidified chamber for 3–5 min to hydrate the spots. Crosslink the slide by ultraviolet (UV) irradiation with 60 mJ in a UV crosslinker. Rehydrate the slide in the humidified chamber and snap dry by heating on the surface of a hot plate for several seconds. Wash the chip in 0.1% SDS for approx 10 s in MilliQ water for approx 10 s and then denature the chip in boiling MilliQ water for approx 1–2 min. After denaturation, immediately immerse the array in ice-cold benzene-free ethanol for several seconds. Remove and allow the chip to dry. Also perform the wash procedure from the SDS to the ice-cold ethanol with the cover slips to be used with the arrays.

3.3.6.2. SAMPLE PREPARATION

1. In parallel, denature 10 μ g each of the representations derived from induced and uninduced cells in the presence of 5 μ g of a random nonamer primer in a total

of 100 μL H_2O . Use either representations ligated to adaptors (*see Subheading 3.2.1.3.*) or with the adaptors cleaved off (*see Subheading 3.2.1.4.*).

2. To each sample, add 12 μL of $10\times$ Klenow buffer and supplement with 33 μM dNTPs, 10 nmol of either Cy3 or Cy5, and 4 U of Klenow fragment. Incubate the reactions at 37°C for 2 h.
3. Combine the two reactions and remove unincorporated nucleotides by centrifugation through a Microcon YM-30 ultracentrifugation column according to the manufacturer's instructions. Wash $3\times$ with 400 λ of EB. Adjust the eluate containing the labeled sample to a volume of 15 μL and a concentration of $3\times$ SSC and 0.2% SDS, and denature by heating to 95°C for 5 min.
4. Carefully place the 15 μL of labeled representations on the array prepared in **Subheading 3.3.6.1.** and slowly place a cover slip on the array. Insert into a hybridization chamber according to the manufacturer's instructions and incubate in the dark at 67°C overnight.
5. After the overnight hybridization, disassemble the hybridization chamber and wash the array at room temperature in 0.1% SDS, $0.2\times$ SSC for 90 s, followed by a 30-s wash in $0.2\times$ SSC and a final 30-s wash in $0.05\times$ SSC.
6. After washing, arrays are imaged in a scanner. Images are saved as TIF files and feature definition and quantitative analysis are performed with either ScanAlyze or Axon GenePix. This analysis yields a text file that is imported into Microsoft Excel or Access for further analysis.

3.3.7. Non-Radioactive Sequencing of PCR Products

Sequence all clones that are found to be differentially expressed based on microarraying (e.g., fluorescence ratio greater than 2). However, do not sequence the Rac clones already identified by dot-blotting in **Subheading 3.3.4.**

1. To obtain sufficient material for nonradioactive sequencing, repeat the PCR from **Subheading 3.3.5.** with only the selected clones as the templates. These PCR samples are checked on a gel and then purified using the Qiaquick PCR purification kit. Determine the concentration of the samples by OD_{260} , which should be at least 25 $\text{ng}/\mu\text{L}$.
2. Perform the sequencing reactions using 8 μL rhodamine dye terminator kit, 2 μL 1.6 μM primer SP6R or T7E, 250 ng ds DNA PCR product, and H_2O to 20 μL . Submit the obtained sequences to BLAST searches.

3.4. Further Characterization of Candidate Genes

It is advisable to reconfirm the expression pattern for the genes which showed altered expression in the microarray experiments by an independent method, such as Northern blot analysis. In the case of less abundant genes, quantitative RT-PCR may be required.

Further analysis of interesting clones will be dependent on the nature of the gene. To further obtain information on the Rac-induced genes, we began to

explore the effects of various growth factors and drugs on the transcription of these genes. We used reagents known to activate or to interfere with previously characterized signaling pathways of Rac. For example, the transcription levels of candidate genes can be compared between resting cells and cells stimulated with PDGF or EGF. Furthermore, Rac is known to activate the p38 MAP kinases, and the drug SB202190 is known to be a selective inhibitor of these kinases (18). The transcription of candidate genes can be compared between cells induced for Rac and either left untreated or treated with SB202190. These experiments are also performed using the microarray technique, allowing simultaneous monitoring of the effect of these reagents on the genes identified by cDNA-RDA.

4. Notes

1. A major challenge in cDNA-RDA experiments is the high risk of contamination, since cDNA-RDA is able to amplify very small differences between two representations. Thus, while performing cDNA-RDA, the use of gloves, sterile filter tips, single-use individually wrapped pipets, and sterile single-use plasticware is highly recommended. Be aware that not all types of plastic withstand the phenol/chloroform solutions used. Therefore, use polypropylene instead of polystyrene tubes. Also, all reagents are used only for cDNA-RDA.
2. To prevent losing sequences later during preparation of the representations (see Subheading 3.2.1.), one can design specific primers for the preparation of cDNAs. Short or unusually composed mRNA sequences may contain either none or only one *DpnII* site. To avoid their loss, use primers with a *DpnII* site added at the 5' end instead of standard random or oligodT primers. Using large amounts of cells derived from an inducible expression system may not be possible in all cases. When starting with smaller amounts of cells (10^6), the Oligotex kit (Qiagen) is recommended for mRNA isolation. If working with even smaller amounts of defined starting material or tissue samples, it may be necessary to use specialized methods during the preparation of representations, as discussed elsewhere (12,6,19). When the samples to be used as tester or driver need to be pooled (e.g., progenitor cells of healthy individuals), it is advisable to avoid pooling the cells or the mRNAs, but to prepare in parallel a representation from each individually and then to combine them.
3. Incubate in an incubator rather than in a water bath or a thermoblock. An incubator is preferred to avoid evaporation of the solution and recondensation on the inner surface of the lid.
4. Be careful not to lose minute amounts of dried DNA in handling. This is a major source of low yields in the following PCR amplification steps. It is also important not to over-dry the DNA pellets, because they become more difficult to redissolve.
5. It is essential to reproducibly obtain representations before starting cDNA-RDA. If a typical yield of approx 150 µg from 0.4 µg template is not obtained, this step must be optimized (such as by preparing new buffers, new cDNAs and/or mRNAs).

6. Depending on the level of induction, the induced gene itself may become a major part of the difference product, when the induced sample is employed as the tester to identify upregulated genes. This can be suppressed by adding this particular transcript back to the driver during both rounds of cDNA-RDA. Although others have recommended to substitution of up to 50% of the driver by the gene (6), we would advise more caution in substituting approx 10% of the driver. An optimal ratio can also be determined experimentally by titration. The Southern blot analysis described in **Subheading 3.3.1.** may not be quantitative enough to detect whether the addition had an effect on the composition of the DP2. However, the difference should be notable in the percentage of individual clones identified by dot-blotting (*see Subheading 3.3.4.*) as being the induced gene.
7. There are only a few ways to judge the quality of the cDNA-RDA experiment during the procedure. It is essential, however, that the yields and amplification rates described throughout the protocol are achieved. Also, digests should result in downward shifts of band patterns in agarose gels (*see Subheading 3.2.1.4., step 3*). Finally, equal aliquots of driver and tester representations, DP1, and DP2 can be run together on a gel (to perform the Southern blot analysis described in **Subheading 3.3.1.**). The observation of appearing or disappearing bands from representation to DP1 and DP2 may indicate that the cDNA-RDA has worked. However, a constant band pattern does not necessarily indicate that the cDNA-RDA has failed. The best method is to proceed with the Southern blot analysis and use its outcome as described in **Subheading 3.3.1.** to judge the quality of the cDNA-RDA experiment.
8. We found that inclusion of 12% DMSO in this PCR buffer significantly increased the yield, and addition of Triton X-100 had no effect on the yield and reduced the clarity of the run on an agarose gel.

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Review

The role of Rho GTPases in disease development

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Abstract

The functionality and efficacy of Rho GTPase signaling is pivotal for a plethora of biological processes. Due to the integral nature of these molecules, the dysregulation of their activities can result in diverse aberrant phenotypes. Dysregulation can, as will be described below, be based on an altered signaling strength on the level of a specific regulator or that of the respective GTPase itself. Alternatively, effector pathways emanating from a specific Rho GTPase may be under- or overactivated. In this review, we address the role of the Rho-type GTPases as a subfamily of the Ras-superfamily of small GTP-binding proteins in the development of various disease phenotypes. The steadily growing list of genetic alterations that specifically impinge on proper Rho GTPase function corresponds to pathological categories such as cancer progression, mental disabilities and a group of quite diverse and unrelated disorders. We will provide an overview of disease-rendering mutations in genes that have been positively correlated with Rho GTPase signaling and will discuss the cellular and molecular mechanisms that may be affected by them. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Rho GTPase; Signaling; Disease; Cancer; Neurological disorder

1. Introduction

The family of Rho GTPases comprise a large subfamily of the Ras-superfamily of GTPases. Among all Rho GTPases, Rac1 (Ras-related C3 botulinum toxin substrate 1), Cdc42 (cell division cycle 42) and RhoA (Ras homologous member A) have been studied most extensively. Through the work of many laboratories the role, that Rho GTPases play in cellular processes as diverse as polarization, cell-cell and cell-matrix adhesion, membrane trafficking, cytoskeletal and transcriptional regulation and cell proliferation has made them a group of crucial regulators with a very general relevance (comprehensively reviewed in Hall, 1998; Van Aelst and D'Souza-Schorey, 1997).

As is the case for small GTPases in general, Rho GTPases are guanine nucleotide binding proteins, which cycle between an active GTP-bound and an inactive GDP-bound

state, and are subject to distinct control mechanisms. In the inactive state, Rho GTPases are associated with a class of negative regulators, the Rho GDP dissociation inhibitors (GDIs), that stabilize the GDP-bound form of the GTPase and sequester them in the cytoplasm. Their active state is promoted by positive regulators called GDP/GTP exchange factors (GEFs) that (a) tether a given GTPase to a distinct subcellular location and (b) by virtue of their signature tandem Dbl homology (DH)/pleckstrin homology (PH) domain exchange GDP moieties associated with the inactive GTPases for GTP. As a consequence, a conformational switch is induced. This in turn renders the GTPase active and allows it to initiate a productive signaling complex with one of several effector proteins. This instigates an information flow to different cellular destinations via different molecular pathways with different physiological outcomes. The active GTP-bound state is counteracted by negative regulators, the GTPase activating proteins (GAPs), that catalyze the intrinsic ability of a small GTPase to hydrolyze the bound GTP-moiety to GDP (hence the name guanosine tri-phosphatases). Thus, effector binding is reversed and signaling activity halted, causing the biochemical system to come full circle. Understanding this biochemical basis for the function of GTPases has greatly benefited research and lead to the development of constitutively active (GTPase-deficient) and dominant negative (nucleotide

Abbreviations: ALS, amyotrophic lateral sclerosis; CDK, cyclin-dependent kinase; DH, Dbl homology; Dia, diaphanous; ECM, extracellular matrix; ERM, ezrin/radixin/moesin; FGD, faciogenital dysplasia; GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; MRLC, myosin regulatory light chain; MRX, X-chromosome linked mental retardation; PAK, p21 activating kinase; PAR, partitioning defective; PH, pleckstrin homology; ROCK, Rho-associated kinase; WASP, Wiskott-Aldrich syndrome protein

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exchange-defective) mutants that lock a respective GTPase in the GTP- or GDP-bound state. The introduction of such mutants into diverse experimental systems allows for either overactivation or functional deletion of a specific GTPase.

There is a growing list of disease-causing mutations in genes that have been associated with Rho GTPase signaling by means of functional prediction or insights obtained by direct biochemical analysis. These include GEFs, GAPs and effector proteins that appear to be part of quite diverse signaling networks. Surprisingly, though, aberrations in only a single gene encoding a Rho GTPase itself, namely the RhoH gene, have been described thus far to be a putative cause of lymphoma development (see below). Other mutations that may inactivate a Rho gene or lead to an overactive version of the resulting protein due to a lack of extensive screening or functional redundancy have either escaped detection or simply are lethal. This latter possibility is underscored by the fact, that mouse embryos whose Rac1 or Cdc42 genes have been deleted by gene-targeted mutation die early in development (Sugihara et al., 1998; Chen et al., 2000). It may also reflect the multifunctional nature of Rho GTPases. Loss-of-function or constitutive gain-of-function mutations in many Rho GTPases thus may interfere with a number of different cellular processes. Based on our current understanding and dependent on the precise physiological circumstances and cell-types under investigation, a single Rho GTPase can affect a diverse array of phenomena implicated in a cell's specific biology. In addition, there is also continued speculation that Rho-type GTPases need to cycle between their active and inactive states in order to exert their complete physiological potential (discussed by Symons and Settleman, 2000).

On the other hand, it is likely that regulators and effectors of Rho GTPases are expressed and act in a more specific manner, be it in the context of a specific cell-type, tissue-type or developmental process. Genetic loss-of-function mutations in these regulators or effectors, even in form of a germline mutation, may result in a weaker impairment than loss of the respective GTPase itself. The continuing revelation of novel genetic lesions in genes encoding Rho regulators and effectors fully supports this possibility.

The following sections summarize examples of disease processes whose underlying genetic alterations affect the normal function and regulation of Rho GTPases. We examine the importance of such mutations in cancer progression, mental disabilities and other disorders.

2. Rho GTPases in cancer progression

The evidence that directly implicates aberrant Rho-signaling activity in cancer has been obtained either by means of mutations uncovered in various genes encoding Rho-signaling components, or by screening and interference protocols that focus on specific aspects of cancer biology. For a detailed summary of the biological understanding of

the pivotal role of Rho-type GTPases in cancer-related processes such as cell-proliferation, migration, invasion and metastasis, we refer the reader to some excellent recent reviews (Symons, 1996; Schmitz et al., 2000; Price and Collard, 2001; Pruitt and Der, 2001). See also Fig. 1.

2.1. Rho GTPases in transformation

In contrast to the 'classical' oncogenic Ras proteins, such as N-Ras, H-Ras and K-Ras, that are frequently mutated in human cancers (Bos, 1988), to date only a single sequence alteration has been detected in a gene encoding a Rho GTPase. In a set of patients diagnosed with non-Hodgkin's lymphoma a t(3;4)(q27;p11–13) translocation was found to be responsible for the pathogenic progression of the disease. Upon closer examination of this locus, a genetic fusion of a gene encoding the newly designated RhoH/TFE GTPase with the LAZ3/BCL3 gene was detected. While a single distinct transcript of the fused loci could be amplified by reverse transcriptase–polymerase chain reaction, it remains elusive whether the promotion of the leukemic aberration is in some way attributable to the RhoH portion of the fusion product (Preudhomme et al., 2000). However, the possibility of RhoH as a player in tumorigenesis has more recently been strengthened by the finding that the RhoH locus is subject to an aberrant hypermutation activity present in B cells. It has been speculated that the somatic hypermutation process that normally generates variable (V) regions in immunoglobulin chains can become misdirected to other genomic loci making it a causative mechanism for about 50% of diffuse large-cell lymphomas. This misdirected hypermutation activity among other loci also has been demonstrated to target the RhoH gene and in particular its upstream non-coding portion. The latter implies the possibility of a change in transcript and/or protein levels contributing to malignant lymphoblastic alterations (Pasqualucci et al., 2001). Again, it has to be mentioned that the specific cellular and biochemical consequences of a mutated RhoH gene as well as the functions of the normal counterpart still have to be determined.

Despite RhoH being the only example of a Rho-specific mutation in humans thus far, it has been clearly demonstrated that Rho-family members play an important role in Ras-induced transformation. Evidence for this has been provided by experiments utilizing constitutively active and dominant negative mutant forms of Rho GTPases in focus forming assays and their ability to enable growth in soft agar as well as tumor formation in nude mice. These protocols allow growth factor-independent proliferation and contact-independent growth. Different laboratories have shown that the activation of distinct Rho GTPases is an essential step towards the fully transformed phenotype triggered by an activated Ras oncogene. Specifically, a dominant negative form of Rac1 markedly inhibits the focus forming activity invoked by Ras in fibroblasts but cannot interfere with an activated, membrane-targeted version of the Raf-kinase

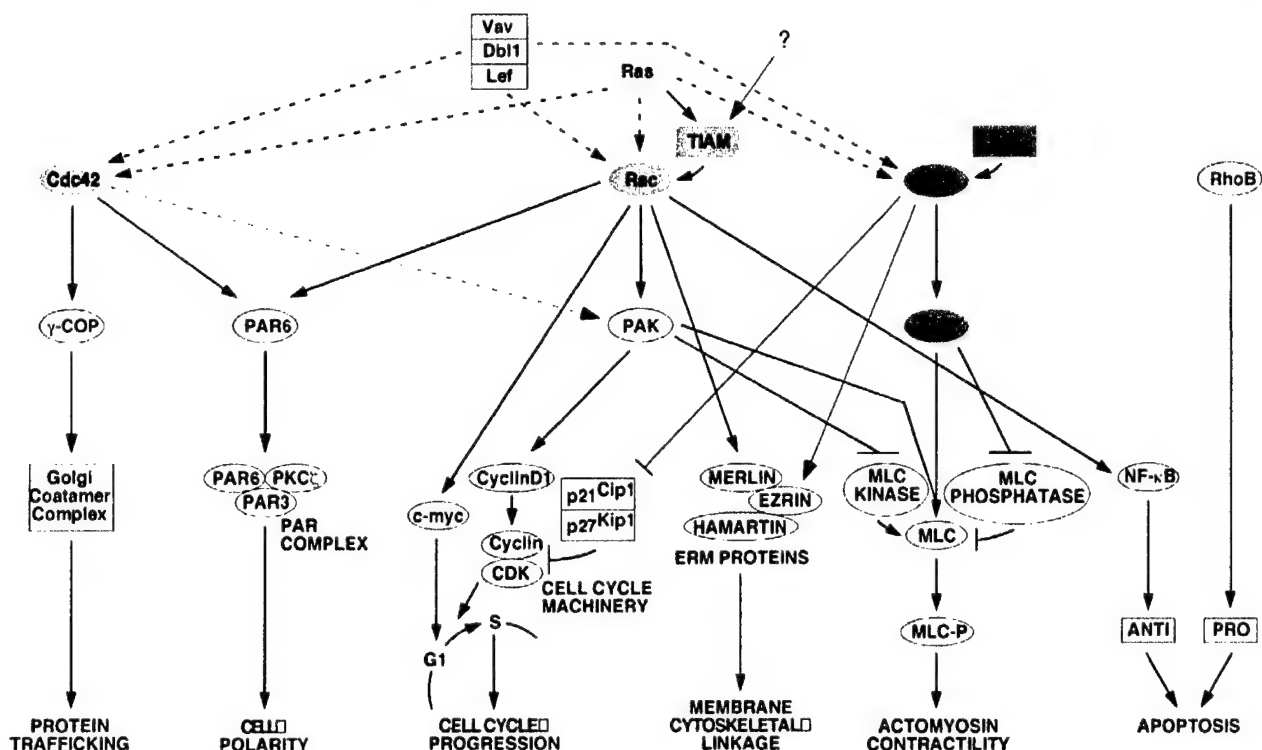


Fig. 1. Potential pathways in which Rho GTPases influence tumorigenic processes. The individual elements depicted in the figure are discussed in Section 2. Boxes, that are highlighted with colors represent small Rho GTPases or molecules that have been found mutated in cancers. Solid lines indicate links, for which unequivocal evidence has been presented.

which acts as the Ras-effector enhancing MAPK activity. In contrast, an activated form of Rac1 significantly enhances the focus forming potential of membrane-targeted Raf (Qiu et al., 1995a). These observations led to the conclusion that Rac is activated by Ras via a Raf-independent pathway. Further studies corroborated these findings and identified RhoA as a second Rho-family GTPase mediating the effects of Ras. Khosravi-Far et al. and Qiu et al. observed that the focus forming activity of a weakly transforming Raf-1 mutant was greatly enhanced when co-introduced together with constitutively active forms of Rac1 or RhoA into fibroblasts. In contrast, the dominant negative forms of Rac1 and RhoA could partially block Ras transformation (Khosravi-Far et al., 1995; Qiu et al., 1995b). In another study, Symons and co-workers added Cdc42 to the list of participants in that they demonstrated that Cdc42 enabled Ras-transformed cells to grow independent of anchorage (Qiu et al., 1997). Additional studies involving Cdc42 suggest that the ability of a GTPase to cycle could be an essential requirement for its transforming potential. In elegant experiments, Lin et al. introduced an F28L mutation into Cdc42, which subjects the GTPase to spontaneous GTP-binding coupled to a wild-type GTP-hydrolysis rate. Cycling in this mutant is therefore greatly accelerated. When stably expressed in fibroblasts, Cdc42^{F28L} yields features of transformed cells including a reduced serum-dependency and anchorage-independency (Lin et al., 1997). Taken together, the genetic interactions summar-

ized above led to the view that Ras not only triggers the activities of the Raf-MAPKK-MAPK cascade but also acts through an alternative route that stimulates Rho-type GTPases which in turn contribute as ancillary factors to the fully transformed phenotype. Furthermore, a picture emerged in which each of the investigated Rho GTPases appears to contribute to a different aspect of the transformed phenotype.

In contrast to the failure to localize mutations in Rho GTPase genes (with the exception of RhoH), there is a growing literature reporting transcriptionally upregulated levels of particular Rho proteins in many types of cancers including those as genetically diverse as those occurring in colon, breast, lung and pancreas (Suwa et al., 1998; Fritz et al., 1999; Jordan et al., 1999; Clark et al., 2000; Schnelzer et al., 2000; van Golen et al., 2000; Kamai et al., 2001). The findings of Jordan and colleagues in particular describe the overexpression of a spliced isoform of Rac1, namely Rac1B, in colorectal tumors, indicating the possibility that oncogenic regulation of Rho GTPases can also occur on the level of mRNA-processing (Jordan et al., 1999; Schnelzer et al., 2000). Recent genomic analysis, combining a protocol selecting for highly metastatic melanoma cells in mice with microarray analysis identified the RhoC gene as a promoter of metastatic behavior. Subsequently, directed expression studies verified this finding (Clark et al., 2000; van Golen et al., 2000). Whether this observation will hold true in

human tissue systems as well, and what the concrete cellular consequences of this phenomenon are, await to be seen. It is noteworthy, however, that, in ductal adenocarcinomas of the pancreas as well as in inflammatory breast cancer cells, RhoC expression was shown to be upregulated (Suwa et al., 1998; van Golen et al., 2000). An intriguing finding is that of the novel Cdc42-like GTPase Wrch-1 as a Wnt-1 transcriptional target (Tao et al., 2001). The Wnt signaling pathway, mostly through mutational analysis of the Wnt-family genes as well as the APC and β -catenin genes, has been implicated in tumorigenesis (Peifer and Polakis, 2000; Polakis, 2000). The contribution of Wrch-1 to the list of Wnt-responsive genes may help understand the mechanisms employed by Wnt-signaling to induce morphological changes, interfere with cell–cell adhesion and cell–extracellular matrix interactions and other phenomena. It also raises the interesting possibility of Rho GTPases functioning as a general class of mediators in multiple oncogenic pathways.

While all these changes in transcript and protein levels are likely to correlate with an elevation in signaling activity *in vivo*, there are few studies that directly assess the activation state of particular Rho GTPases under transforming conditions. One study that does address this issue is the one by Mira and colleagues who were able to monitor an increase in Rac3 activity in transformed and highly proliferative breast epithelial cells derived from human cancer samples (Mira et al., 2000).

2.2. Rho GTPase regulation by upstream activators

As another line of evidence that implicates the Rho-family of GTPases in certain aspects of cancer development, members of the Dbl-family of Rho-GEFs have been classified as oncoproteins. Again, this classification is mainly based on the classical transformation assays mentioned above. The Dbl-family contains such well-investigated members as the founding member of the family, Dbl, the Vav, TIAM, Lcf proteins and others. They all share the tandem Dbl homology and PH homology motifs are now a hallmark feature for Rho-specific activator proteins. Distinct Dbl-family members may show either overlapping activity towards more than one of the Rho GTPases, or specifically activate only one of them (see Fig. 1). Activating deletions and point mutations usually in regulatory regions of the molecules evoke their focus forming abilities. For example, in the case of Dbl and Vav, the N-termini of the proto-oncoproteins have been found to repress their catalytic activities. Accordingly, deletion of these N-terminal sequences can relieve this repression and result in a constitutive activation of the GEF function. The structural basis for some of these autoinhibitory mechanisms and their release has been convincingly disclosed in an NMR spectroscopic analysis focusing on the Vav paradigm of Tyr-phosphorylation-mediated activation (Aghazadeh et al., 2000). Stimulation of cells with particular extracellular agents ensues rapid and transient phosphorylation of Vav which in turn abolishes

its autoinhibitory function with a concomitant increase in GEF-activity (for review, see Bustelo, 2000).

Upon closer inspection of the available literature, we noticed that naturally occurring gain-of-function mutations in patient-derived material have so far only been described for Dbl and the more recently identified LARG and TIAM genes. The Dbl oncogene was originally isolated from a human diffuse B-cell lymphoma DNA in a coupled gene transfer/transformation assay (Srivastava et al., 1986), while LARG (leukemia-associated Rho guanine exchange factor) was isolated as a fusion partner of the MLL (mixed lineage leukemia) gene in acute myeloid leukemia (Kourlas et al., 2000). Subsequently, work by two groups produced biochemical evidence for LARG's identity as a Rho activator (Fukuhara et al., 2000; Reuther et al., 2001). The protein's tandem DH/PH domain appears to be specific for RhoA since LARG can activate the Rho-dependent serum response factor (SRF) but not Rac/Cdc42-dependent Jun kinase (JNK) signaling (Reuther et al., 2001). Taya et al., in a recent study, revealed that the PDZ domain of LARG associates with the carboxyterminal tail of the insulin-like growth factor-1 (IGF-1) receptor (Taya et al., 2001). Elaborating on this finding, the authors further produce evidence that IGF-1 stimulation of epithelial MDCKII cells results in an activation of RhoA and its effector kinase ROCK to give rise to enhanced stress fiber formation. Since the IGF-1 receptor/LARG complex forms constitutively also in the absence of IGF-1 ligand but on the other hand RhoA activity is triggered by exposure to IGF-1, the authors speculate that LARG activation is subject to an as yet unidentified IGF-1-dependent mechanism (Taya et al., 2001). It is also possible, that the RGS (regulator of G-protein signaling) domain harbored by the protein serves to couple G-protein-coupled receptors (GPCRs) and heterotrimeric G proteins of the G α (12) family to Rho-dependent signaling (Fukuhara et al., 2000). These initial biochemical studies, together with the finding that LARG is expressed in all human tissues examined, will surely promote further interest in this molecule.

TIAM (T-cell invasion and metastasis gene) was found in an extensive screen, that was designed to isolate invasion-promoting genes in T cells (Habets et al., 1994). Subsequently, TIAM was demonstrated to display Rac-specific exchange activity (Michiels et al., 1995). In light of earlier experiments that showed Rac to promote the spread of cancer cells and metastasis in nude mice (Qiu et al., 1995a), these data proposed a TIAM/Rac-dependent, invasion-promoting signaling pathway. This opened a different perspective on the role of Rho-proteins in cancer progression, namely their function in metastasis and invasion in aggressive cancer cells. This property of some Rho GTPases has since been reiterated in different experimental settings (e.g. the one described for the finding of RhoC above). In fact, analysis of the TIAM locus in a set of renal-cell carcinomas revealed several mutant TIAM alleles. One of these mutations, A441G, maps to the protein's N-terminal pleckstrin homology domain and could potentially interfere with

the proper membrane localization and functional activity of the protein. In focus forming assays, the A441G mutation can convert TIAM into a transforming molecule which further underlines its relevance in malignant processes (Engers et al., 2000). Interestingly, a putative metastasis suppressor, nm23H1, which was isolated from murine melanomas by subtraction cloning associates with TIAM and seems to negatively regulate cell motility of tumor cells of murine and human origin (Leone et al., 1991; Kantor et al., 1993; MacDonald et al., 1996). This activity correlates with a downregulation of Rac1 (Otsuki et al., 2001). Thus nm23H1 may prove to be an interesting physiological link to TIAM and Rac regulation during invasion and metastasis. It has to be mentioned that depending on the specific cell type under investigation (Hordijk et al., 1997) and the particular ECM composition faced by those cells (Sander et al., 1998), TIAM can also generate an adhesion-promoting effect. In epithelial MDCK cells, its expression may even reverse the loss of E-Cadherin mediated adhesion induced by oncogenic Ras (Hordijk et al., 1997). Therefore, the involvement and regulation of TIAM in invading and metastasizing cancer cells might be more complex than the current data suggest.

2.3. Downstream effectors and effector pathways

What is the nature of the underlying signaling pathways mediating distinct aspects of cellular transformation triggered by Rho GTPases and which are the immediate downstream effectors regulating them?

A necessary requirement for a cell to transit from a normal to a transformed state is the dysregulation of its cell cycle machinery. From earlier observations, it became evident that cyclin D1, as a key factor required for the G1/S transition whose levels oscillate throughout the cell cycle, is transcriptionally upregulated by the Ras-induced Raf/MEK/ERK pathway (Cheng et al., 1998; Kerkhoff and Rapp, 1998). Subsequently, it has been demonstrated that Rac can also stimulate activity at the cyclin D1 promoter (Westwick et al., 1997; Gille and Downward, 1999). Cyclin D1, as a functional consequence of its enhanced synthesis, teams up with its cognate cyclin-dependent kinase partners CDK4 and CDK6. The major substrate for the phosphorylation-activity of the complex is the Retinoblastoma protein whose subsequent degradation triggers progression through the G1-phase of the cell cycle (Sherr and Roberts, 1999). It is worth noting, that a survey of human breast cancer etiology in patients has revealed the cyclin D1 gene to be amplified in 20% of the cases examined (Dickson et al., 1995). Moreover, cyclin D1 protein levels appeared to be elevated in more than 50% of mammary carcinomas (Bartkova et al., 1994; Gillett et al., 1994; McIntosh et al., 1995). Further corroborating the relevance of this transcriptional target, Yu et al. (2001) observed that cyclin D1-deficient mice are able to resist cancer development induced by the Ras and Neu oncogenes. It is conceivable that Rac could either contribute

to the full oncogenic upregulation of cyclin D1 transcription by Ras or that it leads to a sustained promoter activity. However, Rac can also activate the cyclin D1 gene in Ras-independent pathways. Consistent with this, activation of integrin signaling has recently been demonstrated to modulate the levels of cyclin D1 protein in a Rac-dependent manner in order to trigger proliferation of primary cells in culture. Surprisingly, in this case Rac appears to influence cyclin D1 expression on the translational rather than the transcriptional level (Mettouchi et al., 2001). In a recent publication Welch et al. presented a more refined concept highlighting differential effects of Rho GTPases on cyclin D1 expression (Welsh et al., 2001). Whereas, in their studies Rac1 and Cdc42-emanating mechanisms induced cyclin D1 early in G1-phase of the cell cycle, Rho-activity may ensure sustained cyclin D1 expression throughout G1 in an ERK-dependent fashion. Apart from cyclin D1 as a transcriptional target, the expression of the cell-cycle regulator c-myc, induced by PDGF, has been demonstrated to rely on Rac activity and to occur independently of Ras. Together, this information suggests, that a single Rho GTPase might affect the cell cycle via a number of different pathways (Chiariello et al., 2001).

Rho, on the other hand, seems to impinge on another class of cell cycle regulatory proteins, namely the cyclin/CDK complex inhibitors p21^{Cip1} and p27^{Kip1}. Evidence for this comes from the observations, that (1) Rho activity interferes with p21^{Cip1} in that it suppresses its induction (Olson et al., 1998), and (2) p27^{Kip1} degradation is facilitated by Rho (Weber et al., 1997; Hu et al., 1999). Hence, as in the case of cyclin D1 upregulation, these Rho-specific phenomena lead to a stabilization of cyclin/CDK activity, which consequently is thought to accelerate progression through the cell cycle.

To date, it remains unresolved, which of the many effector molecules of the Rho-family GTPases unambiguously relay an oncogenic potential in vivo and which of the activated signaling pathways are the ones responsible. There have been no activity-modifying mutations described in any of the known Rho GTPase effector proteins in cancerous cells. One of the candidates relaying the Rac signal to the cell cycle machinery in order to achieve transformation, however, is the Ste20-like p21 PAK-kinase. PAK kinases were first identified by Manser et al. who performed biochemical overlay assays with the intention to isolate novel Rac1 and Cdc42 effector proteins (Manser et al., 1994). A catalytically inactive form of PAK can effectively suppress Ras-induced transformation in some cell lines (Tang et al., 1997) and, it has been shown that PAK activity correlates with cyclin D1 promoter induction (Joyce et al., 1999). Similarly, the function of Rac3 in the proliferation of breast tumor cells appears to correlate with increased PAK activity (Mira et al., 2000). PAK, apart from its correlation to cell proliferative events, also has been related to aspects of microfilament reorganization and invasiveness in breast cancer cells. In this setting, a kinase-dead version of PAK1

can suppress the motile and invasive phenotypes of otherwise highly invasive human MDA-MB435 cells (Adam et al., 2000).

Although these data suggest a role for PAK in tumorigenesis/metastasis, it remains unclear as to whether PAK is dependent on Rac-activity to exert its effect. It has been demonstrated that PAK-independent pathways emanating from Rac are likely to participate in promotion of Rac's transforming potential. It also has been shown that Rac-mutants engineered to no longer bind PAK, but that still tether other effectors, can persist to evoke transformation of fibroblasts (Joneson et al., 1996; Lamarche et al., 1996; Westwick et al., 1997). This is suggestive of the notion, that Rac can target the cell cycle apparatus through PAK-dependent or PAK-independent pathways or by multiple pathways operating simultaneously in a given cell.

The ROCK effector-kinases have been proposed to convey Rho's transforming ability. ROCK-type kinases serve as regulators of the actomyosin network in that they promote phosphorylation of the regulatory light chain of myosin in a Rho-dependent manner. This in turn stimulates contractility of actin filaments, which is the basis of cell motility. A constitutively active version of the kinase can synergize with activated Raf in conventional transformation assays (Sahai et al., 1999). An additional and intriguing piece of evidence for ROCK's role in tumorigenesis has been provided by Narumiya and colleagues who developed and successfully applied a ROCK-specific inhibitor (Y-27632). Highly metastatic Rat MM1 hepatoma cells are able to transmigrate through a mesothelial cell monolayer in a serum and specifically lyso-phosphatidic acid-dependent manner. This migratory behavior is enhanced by transfection with a dominant positive form of ROCK, while conversely, it is inhibited by a ROCK mutant acting as a dominant negative version of the kinase. Treatment with Y-27632 can block Rho-mediated invasion in MM1 cell cultures and, moreover, can interfere with the dissemination of MM1 cells when implanted into the peritoneal cavity of syngeneic rats (Itoh et al., 1999). Y-27632 also interferes with the transforming activities of RhoA itself and the ones of the Rho-activators Dbl and Net (Sahai et al., 1999). In the first experiments with human cells, Y-27632 was able to partially abolish the anchorage-independent growth of two colorectal carcinoma cell lines and in another assay inhibited human prostate cancer cells from disseminating when introduced into immune-compromised mice (Somlyo et al., 2000). Whether this inhibitor will prove to be genuinely ROCK-specific and whether the data from these animal experiments can be extrapolated to the invasion of human metastatic cells in human tissues has to be shown by further careful investigation.

Recently, it was found that ROCK-dependent phosphorylation of the Ezrin/Radixin/Moesin (ERM)-family member Ezrin is a necessary requirement in the transformation process induced by oncogenic Dbl-proteins (Tran Quang et al., 2000). ERM-proteins are thought to link the actin cytoskeleton to membrane regions undergoing dynamic morpho-

logical changes. Net or Dbl oncogene expression can interfere with the normal cell-cell contact inhibitory effects on proliferation, which may contribute to their oncogenic potentials. Introduction of an Ezrin mutant that does not serve as a ROCK-substrate anymore may re-establish a contact-inhibitory mechanism under those circumstances. This clearly is indicative of the possibility, that Rho/ROCK signaling constitutes the link between Dbl- and Net-activity as a malignant stimulus and an ERM-protein function at the membrane as one of its outputs. The observation, that ROCK-dependent Ezrin phosphorylation can be blocked by Y-27632 addition nicely supplemented this connection (Tran Quang et al., 2000). Ezrin also has been found to interact with Hamartin, the gene product of the TSC1 tumor suppressor gene locus (Lamb et al., 2000) as well as RhoGDI and the Dbl oncoprotein (Takahashi et al., 1997, 1998). Together, these insights strongly emphasize the importance of Rho/ROCK mediated signaling in cancer progression.

Another correlation between Rho GTPase signaling and an ERM-like protein is the one that has been established more recently between Rac and Merlin by Shaw and co-workers (Shaw et al., 2001). Merlin is the product of the NF2 gene, mutations in which predispose humans and mice to the development of Neurofibromatosis type II (NF2). Since mutations in the NF2 gene that abolish Merlin synthesis are facilitating or causing tumor formation, NF2 has been defined as a tumor suppressor gene (Gutmann et al., 1997; Gusella et al., 1999). In an endeavor to shed light on the molecular network NF2/Merlin is embedded in, Shaw et al. have produced evidence that the functional state of Merlin is controlled by Rac. The expression of constitutively active Rac or its activators Dbl and Tiam promotes the phosphorylation of Merlin at the critical C-terminal Ser-518 residue. This modification is thought to inhibit the 'closed' conformation of the molecule that relies on the head-to-tail interaction between N- and C-terminal residues within the Merlin protein and rather promotes its 'open' conformation. Since it is the closed state that exhibits a negative growth regulatory function in vitro and in vivo (Sherman et al., 1997), the release of its inhibitory functions by activated Rac may provide a contribution to Rac's tumorigenic potential. This possibility has been strengthened by additional experimentation that showed Rac-induced transformation to be drastically reduced by concomitant expression of Merlin. NF2-deficient fibroblasts also display features that strongly resemble those elicited by Rac, such as membrane ruffling and an increase in the number of intracellular vesicles (Shaw et al., 2001). These data taken together suggest an interesting model. Merlin might be linking events at the plasma membrane with the cell cycle machinery and in particular cell cycle-promoting cyclin D1 levels. In a setting, where no functional Merlin protein is present like in NF2 mutant cells, this block is genetically abolished. Alternatively, in cases where a Rac-dependent mechanism interferes with the growth-inhibitory properties of wild-type Merlin, its function is biochemically disrupted. Although speculative, this scenario could offer a

partial explanation for the Rac-provoked cell cycle stimulation.

A potentially oncogenic mechanism involving a Rho GTPase and impacting on vesicle trafficking has recently brought to light (Wu et al., 2000). Whereas Rho GTPases have been implicated in various steps of membrane trafficking (for review see Ridley, 2001), the significance for this in oncogenic processes remains ill-understood. Cdc42 has been implicated by Wu et al. in the functional modulation of the Golgi coatamer complex. The coatamer complex serves to shuttle cargo from the endoplasmic reticulum to the Golgi apparatus and Cdc42 directly associates with the γ -COP subunit (Wu et al., 2000). The same authors found that Cdc42 has to target γ -COP in order to generate the transformed phenotype characteristic of the 'fast cycling' Cdc42^{F28L} mutant mentioned above. This observation provides the first clue for the involvement of membrane trafficking in cellular transformation (Wu et al., 2000). It was known earlier, that Cdc42 localizes to the Golgi compartment and that it affects several transport steps such as the exit of apical and basolateral proteins from the trans-Golgi network and the endocytic transport to the basolateral plasma membrane in polarized cells (Erickson et al., 1996; Kroschewski et al., 1999; Musch et al., 2001). While the precise consequences of the Cdc42/ γ -COP interaction are not well understood, it may be a first molecular link for Cdc42's association with the Golgi compartment and its local effects therein.

Another door that just has been opened concerns the role of Rho GTPases in the establishment of cellular polarity. While the general relevance of Rho GTPases in these processes has been demonstrated, the discovery of the PAR6 protein as a Rac1 and Cdc42 effector contributes a first molecular insight. PAR6 is a constituent of the Par-6/Par-3/PKC ζ complex that is vital for the establishment of cellular polarity in diverse systems (Joberty, 2000 #167; Johansson, 2000 #170; Lin, 2000 #168; Qiu, 2000 #138). In mammalian cells, PAR3, PAR6 and PKC ζ are associated with tight junctional structures and Rac1/Cdc42 engagement of the PAR3/PAR6 complex is thought to participate in tight junction formation in part likely by inducing PKC ζ activity (Joberty et al., 2000; Qiu et al., 2000). Overexpression of a kinase-dead PKC ζ version in MDCK cells causes mislocalization of PAR3 correlating with a severe impairment of tight junctional biogenesis (Suzuki et al., 2001). Moreover, ectopically expressed PAR6 enhances the transforming potential of Rac1 (Qiu, 2000 #138). These experiments clearly indicate that the PAR3/PAR6/PKC ζ complex is sensitive to changes in the ratio of its components and to ectopically altered levels of its kinase activity. Since tight junctional integrity is required for the maintenance of cell polarity and proper polarity is abolished in transformed cells, targeting of the Par-6/Par-3/PKC ζ complex by activated Rac1 or Cdc42 could contribute to malignant transformation. The activation of atypical PKC isoforms including PKC ζ previously has been correlated to the

control of cell growth and survival. Ectopic amounts of PKC ζ can counteract apoptotic signals and experimental down-regulation of PKC ζ levels and activity impair cell proliferation and activation of NF- κ B transcription which in many situations prevents apoptosis (Berra et al., 1993; Dominguez et al., 1993; Diaz-Meco et al., 1996).

It has been demonstrated that the cell survival machinery appears to be directly affected by Rho GTPases. Like oncogenic Ras (Finco et al., 1997; Mayo et al., 1997), Rac, Cdc42 and Rho positively regulate the transcription at NF- κ B-dependent promoters (Sulciner et al., 1996; Perona et al., 1997) and thus may prevent cells driven to a transformed state from undergoing apoptosis. NF- κ B also appears to be required for the transforming abilities of the RhoA and Cdc42 activators Dbl and Dbs (Whitehead et al., 1999). These pathways can also interfere with Ras-induced apoptosis (Joneson and Bar-Sagi, 1999) and the expression of dominant active Rac can prevent suspension-induced apoptosis ('anoikis') of epithelial cells (Coniglio et al., 2001) as well as apoptosis triggered by serum deprivation of fibroblastic cells (Ruggieri et al., 2001). It will be interesting to see, whether PKC ζ activation in polarity determining complexes by Rac1 and/or Cdc42 will connect through a distinct signaling pathway to antiapoptotic and proliferative events like e.g. activation of NF- κ B. Of note is the observation that Rac2-deficient cells derived from gene targeted mice display significantly reduced survival in the presence of growth factors as compared to control cells. This property, furthermore, has been correlated with a failure to induce the survival factors Akt and BAD/Bcl-XL (Yang et al., 2000).

Targeted deletion and transgenic expression experiments in mice revealed diverse roles also for Rho GTPase genes and their products in mediating apoptosis and cell survival in a context-dependent manner (Cleverley et al., 2000; Costello et al., 2000; Liu et al., 2001a). Mutant mouse embryo fibroblasts (MEFs) that have been derived from RhoB-deficient mice when transformed with the H-Ras and adenovirus E1A oncogenes displayed a significantly elevated resistance to apoptosis after being exposed to DNA-damaging reagents as compared to non-targeted cells. It appears that RhoB has taken on a unique role among the Rho GTPases. In contrast to the developmental necessity of the Rac1 and Cdc42 genes and their products, the RhoB gene is dispensable for development and normal physiological aspects of mouse biology (Sugihara et al., 1998; Chen et al., 2000; Liu et al., 2001b). However, the proapoptotic function that has been ascribed to RhoB may be required under conditions that generate cellular stress as exemplified by the exposure to DNA- and microtubule damaging compounds or under cell transforming circumstances (Liu et al., 2001a,b). In fact, the relatively high turnover rates the RhoB protein is subjected to often are counteracted by transcription of the RhoB gene (Jahner and Hunter, 1991; Fritz et al., 1995). The currently available data hence strongly argue for a proapoptotic function for RhoB that eliminates damaged cells with a potential to elude normal cell cycle control and thus to become a cancer-

ous hazard for the organism. This property clearly distinguishes RhoB physiology from the anti-apoptotic aspects of Rac1.

In contrast, in hemopoietic cells Rho has clearly been correlated with cell survival signaling (Costello et al., 2000). The thymocyte-specific *lck* promoter was used to drive the expression of the Rho-inhibitor C3 (bacterial toxin C3 transferase from *Clostridium botulinum*). C3 selectively ADP-ribosylates and inactivates RhoA, B and C (Boquet, 1999). Under these conditions, pre-T cells that have not undergone β selection to assemble $\nu\beta$ -chains into functional pre-T cell receptors (TCRs) are subject to massive apoptosis. This cell death phenomenon is abrogated in a p53 loss of function situation suggesting a p53-dependent mechanism that is governed by Rho-activity. After formation of functional pre-T cell receptors, however, Rho appears to promote survival via a p53-independent and BCL-2-sensitive pathway (Costello et al., 2000). Another study by Cleverley et al. shows that the same mice, when 4 to 8 months old, develop aggressive thymic lymphoblastic lymphomas (Cleverley et al., 2000). Analysis of the tumors revealed a lack of heterogeneity in the $\nu\beta$ -chain of the T-cell receptor (TCR) complex which led the authors to hypothesize a monoclonal origin of the malignant cells. Since *lck*-driven C3 expression alone does not ensue tumor formation *per se*, it is likely that an additional genetic alteration underlies the observed clonal expansion. Given that all 3 Rho isoforms are interfered with by C3 and taken in particular the RhoB functions described above into consideration, it is conceivable, that concomitant inactivation of RhoB could favor tumor development by inhibiting apoptosis in malignant clones.

In summary, the significance of Rho GTPase signaling impinges on various aspects of oncogenesis (see Fig. 1). As exemplified in the aforementioned, cell cycle progression in tumor cells, their adhesive properties, migratory and invasive behavior and escape from apoptotic extinction all seem to be affected by Rho GTPase activity. Whereas a scaffold emerges in which a particular Rho GTPase is linked to specific physiological effects, defined signaling pathways still need to be worked out. As is the case with Y-37632 as a ROCK inhibitor, other participants in specific signaling pathways may serve as drug targets to inhibit malignant processes that depend on Rho GTPase activity. In addition, given, that dysregulations provoked by Rho GTPases phenocopy cancerous aspects of oncogenesis in general, it is tempting to speculate that Rho signaling might be contributing to other oncogenic pathways as well. The identification of *Wrch-1* as a transcriptional target of oncogenic Wnt signaling is a first example for such interdependencies.

3. An emerging role for Rho GTPases in neurodegenerative disorders

Rho GTPases are currently gaining increasing attention

for their involvement in different classes of neurodegenerative disorders that reflect vital functions of Rho GTPases in diverse aspects of the nervous system. Over the past few years, Rho GTPases have been implicated in neuronal processes including neuronal migration and polarization, axon guidance and dendrite formation, as well as synaptic organization and plasticity (comprehensively reviewed in (Luo, 2000)). Given the large number of GEFs and GAPs shown to be functional and/or expressed in the nervous system, and others that are predictable on the basis of the available genome sequences, it is likely that Rho GTPase activity is intrinsic to various signaling pathways involved in the regulation of neuronal processes. Many of these pathways will be responsive to extracellular cues and stimuli to evoke cytoskeletal rearrangements, which underlie detectable morphological adjustments in cells of the nervous system (Fig. 2).

One complex of quite heterogeneous neuropathological disorders constitutes the nonsyndromic, X-chromosome linked forms of mental retardation (commonly referred to as MRX or XLMR). MRX affects approximately 1 in 500 males and represents about 25% of all genetically manifested cases of mental retardation. The only feature in individuals affected by MRX is an impairment of their cognitive functions. While no gross anatomical alterations in brain structures have been observed in MRX-inflicted individuals, closer histological inspection has revealed that the hippocampus and certain cerebellar ventricles often are increased in size. In contrast, the cerebral cortex often appears reduced in size when compared to unaffected control tissue (Reiss et al., 1991; Reiss et al., 1994). Detailed microscopic analysis showed that the dendritic spines in the affected regions are thinner and more elongated in MRX-patients. Furthermore, the synaptic contacts they establish are more reminiscent of those made by immature spines (Rudelli et al., 1985; Hinton et al., 1991). Spine synapses are considered to relay the majority of functional excitatory synaptic communication. Moreover, they are regarded as the structures displaying most of the ongoing synaptic plasticity that determines the efficacy of 'learning and memory' processes (Matus, 1999). Moreover, spine morphology is particularly dependent on actin structures and processes that continuously remodel them. These are processes that in many respects require the directed regulation and activity of the Rho-family of GTPases (Luo, 2000).

Through means of positional cloning, about ten genes associated with MRX have been identified thus far, offering the first mutational basis to study the specific genetics and biochemistry involved in MRX. Among these, three MRX genes encoding oligophrenin-1, α PIX/Cool-2/ARHGEF6 and PAK3 represent elements of potential Rho GTPase-dependent signaling pathways that are active in neurons. Others, such as IL1RAPL and TM4SF2 being additional identified MRX-genes and FMR1 being associated with Fragile X mental retardation syndrome (FraX) may be physiologically linked to Rho-function.

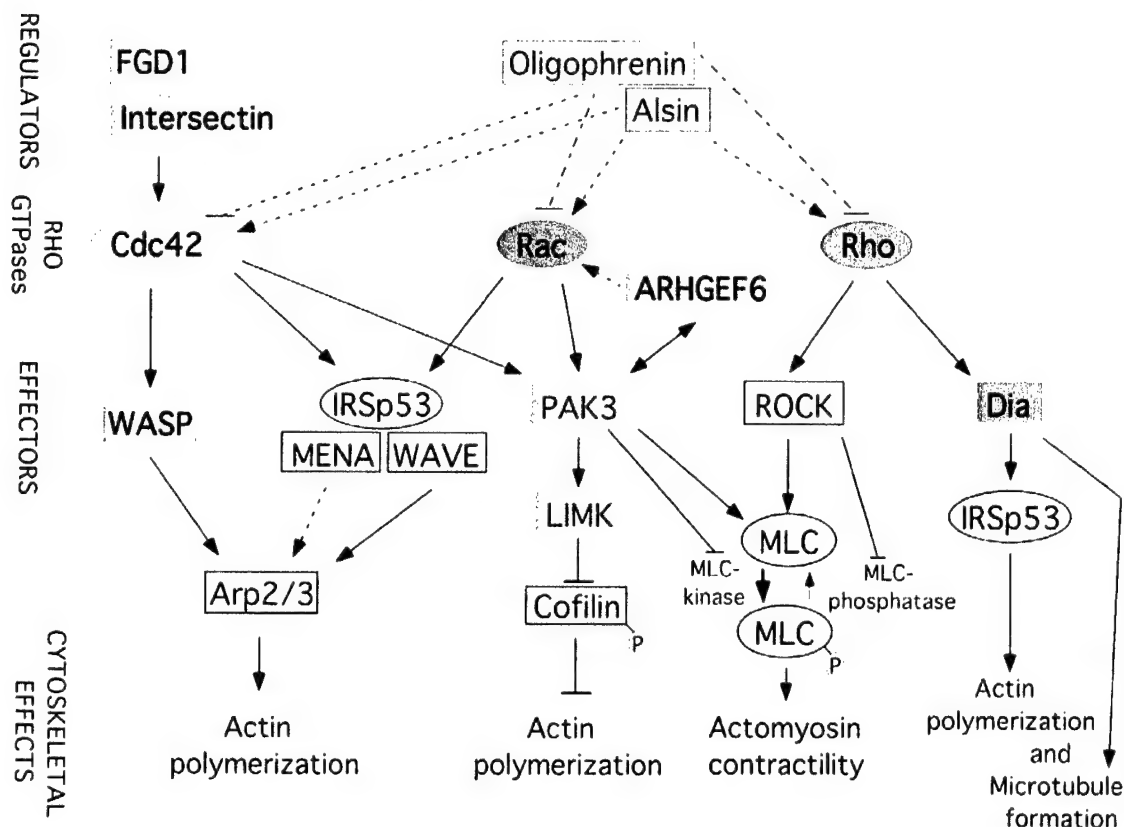


Fig. 2. The putative signaling networks in which Rho GTPase related genes involved in mental retardation and other disorders may affect the cytoskeleton. The specific genes and their products are referred to in Sections 3 and 4.

Besides the genetic lesions underlying various forms of mental retardation, the recent description of mutations in a gene encoding a potential RhoGEF, called alsln, as a cause for amyotrophic lateral sclerosis (ALS) presents another example for the involvement of Rho GTPases in neuronal processes. ALS is a fatal neurological disorder in which motor neurons progressively degenerate. The finding that a putative RhoGEF is mutationally inactivated in this disease suggests that impairment of proper Rho GTPase signaling may be an important step in the etiology of neurodegenerative phenomena.

Finally, another candidate molecule that is being discussed as a disease-related Rho GTPase signaling component is intersectin. The intersectin gene is located on human chromosome 21 and the biochemical properties revealed so far indicate that its protein product could contribute to the neuronal defects observed in Down syndrome.

3.1. Mental retardation-associated genes

3.1.1. Oligophrenin-1

The first Rho-related MRX gene to be identified was the oligophrenin-1 gene. It encodes a putative RhoGAP protein with a canonical RhoGAP catalytic domain, which is likely to have a negative effect on Rho GTPase signaling. Two

different mutations in the oligophrenin-1 gene have been isolated, both of which are supposed to lead to a loss-of-function phenotype (Antonarakis and Van Aelst, 1998; Billuart et al., 1998). So far, it remains incompletely understood as to which of the Rho GTPases is the physiologically relevant target of oligophrenin-1 in vivo although the protein exerts GAP activity towards RhoA, Cdc42 and Rac1 in vitro. This observation emphasizes a role for oligophrenin-1 as a negative regulator of Rho GTPase activity. Nevertheless, more specifically designed experiments will have to address the in vivo substrate preference, subcellular localization and the nature of the protein network it is part of to understand the disease-causing role of the isolated mutations. It is conceivable that oligophrenin-1 also fulfills extra-neuronal functions, since it is expressed in tissues other than the brain, albeit at lower levels (Billuart et al., 1998). However, loss of oligophrenin-1 function in humans does not give rise to any discernible phenotypes in addition to cognitive impairments in affected individuals suggesting that a loss of oligophrenin-1 in other tissues may be compensated for by functional redundancy.

3.1.2. PAK3

A second Rho-related MRX-gene is PAK3. Two different mutations in the PAK3 gene have been isolated in two

MRX-pedigrees. As described earlier, PAK kinases have been defined as Rac1- and Cdc42-specific effector molecules that activate pathways downstream of activated Rho GTPases (Manser et al., 1994, see Fig. 2). PAK3 is highly expressed in MRX-relevant brain regions, namely the cortex and hippocampus, suggesting that PAK3 kinase function is required either for the development of these regions, or for the inherent synaptic plasticity of synaptic spines in specialized regions with particular cognitive tasks (Allen et al., 1998). PAK kinases, according to a current model, are thought to exist in a cytoplasmically dormant state. Upon recruitment by an upstream factor, which could be activated Rac1 or Cdc42, the autoinhibitory interaction between the N-terminal regulatory domain and the C-terminal catalytic domain is disrupted. This switches PAK into an active conformation capable of auto- and substrate phosphorylation (Aghazadeh et al., 1998; Bagrodia and Cerione, 1999; Buchwald et al., 2001). A point mutation present in a multiplex pedigree abrogates the Ser/Thr-directed kinase function by inserting a premature stop-codon (Allen et al., 1998). In another MRX pedigree, the missense mutation R67C has been implicated as a cause for moderate to severe mental retardation. This amino acid substitution resides in the predicted Rho GTPase-interaction motif of PAK3 and hints to the relevance of the GTPase/PAK interaction (Bienvenu et al., 2000). Of note, mutations in the *Drosophila* PAK homolog have been implicated in developmental aspects of the nervous system such as neurogenesis and axonal pathfinding (Melzig et al., 1998; Hing et al., 1999; Newsome et al., 2000). Imperfectly understood downstream signaling pathways relay PAK-activity to the actin cytoskeleton and possibly the nucleus. The identification of the PAK-relevant kinase targets in MRX will greatly contribute to our understanding of the physiological correlation between Rho GTPase activities and cognitive functions.

So what are the molecular components linking PAK to cytoskeletal alterations in neurons? Different proteins have been hypothesized to serve as substrates for PAK's kinase activity in general and particular neuronal situations. PAK, as part of its activation mechanism, phosphorylates itself (Manser et al., 1994). Furthermore, LIMK (or Lim-domain-containing protein kinase) has been identified by Edwards et al. as a potential downstream mediator of PAK (Edwards et al., 1999). Upon phosphorylation by PAK, LIM-kinase in turn phosphorylates, and thus inactivates, the actin depolymerizing factor Cofilin (Arber et al., 1998; Yang et al., 1998). This study suggests that PAK can connect Rac/Cdc42 activity to LIM-kinase in the regulation of actin cytoskeletal dynamics. Interestingly, hemizygosity of the LIM-kinase 1 encoding locus on chromosome 7q11 is correlated with the autosomal William's syndrome, a disorder comprised of a complex of phenotypes which affects about 1 in 20,000 births. Features of this condition include weakened visuospatial constructive cognition and varying severities of mental retardation (Frangiskakis et al., 1996). Another substrate of PAK is MLCK (myosin light chain

kinase), which exerts kinase activity towards the myosin regulatory light chain (MRLC) (Sanders et al., 1999). MRLC-phosphorylation by MLCK is thought to stimulate actin/myosin motor complexes. Since MLCK is inactivated by PAK, this event is believed to counteract actin/myosin motor activity. In addition, MRLC has also been proposed to be a direct PAK target (Sells et al., 1999). Most of these initial biochemical observations have come from the study of fibroblasts or epithelial cell lines. Thus, these different findings will have to be re-evaluated as to their importance in neurons in order to elucidate the relevant mechanisms involving the loss of PAK3 in MRX.

3.1.3. ARHGEF6

Another intriguing addition to the list of MRX-causing mutations is the ARHGEF6 gene discovered by Kutsche and colleagues (Kutsche et al., 2000). The ARHGEF6 gene product is identical to the previously discovered α PIX/Cool-2 protein isolated as a PAK-binding partner by means of biochemical co-purification and yeast-2-hybrid approaches. In fact, a small protein family containing α PIX/Cool-2 itself, β PIX/p85Cool-1 and its smaller splice variant p50Cool-1, has emerged (Bagrodia et al., 1998, 1999; Manser et al., 1998). Interestingly, α PIX/Cool-2 also contains the typical tandem DH/PH motif that marks it as a potential RhoGEF (Fig. 2). The potential for an interaction between α PIX/Cool-2 and PAK is mediated by an SH3 domain in the former and an unconventional SH3-binding site in the latter protein. α PIX/Cool-2, when co-expressed with PAK, can trigger PAK's kinase activity (Bagrodia et al., 1998; Daniels et al., 1999). Attempts to monitor directly the levels of [3 H]GDP released from Rac1 or Cdc42 did not unequivocally reveal an exchange activity towards either of the GTPases. However, in vitro assays, α PIX/Cool-2 can increase the amount of GTP-bound Cdc42, whereas its sibling β PIX/p85Cool-1 can increase GTP levels bound to Rac1. This activity, however, is significantly lower than that of the well-studied RhoGEF Dbl (Bagrodia et al., 1998, 1999; Manser et al., 1998; Daniels et al., 1999). Taken together, the expression and biochemical experiments performed so far suggest that α PIX/Cool-2 can enhance PAK recruitment and activity but do not give an unambiguous answer as to the exact molecular mechanisms governing the GTPase/ α PIX/Cool-2/PAK complex. Most probably, other factors and precise subcellular conditions dictate the function of this complex in various in vivo settings. As in the case of PAK3, comprehension of the concrete cellular effects of the mutations found in ARHGEF6 requires further study.

3.1.4. IL1RAPL, TM4SF2 and FMRP

Other than the genes described above that are direct regulators or effectors of Rho GTPases, there is a group of mental retardation genes that are not evident Rho-signaling components but may still be indirectly linked to the function of Rho GTPases. IL1RAPL, TM4SF2 and FMRP code for a

novel member of the interleukin-1 (IL-1) receptor family, a protein of the tetraspanin family of membrane proteins and an RNA-binding protein, respectively (Carrie et al., 1999; Zemni et al., 2000). It has been reported that IL-1, an inflammatory cytokine, can stimulate Cdc42 through its cognate IL-1 RI and RII receptors in fibroblasts. As a consequence, Rac and Rho GTPases also become activated. In fact, Cdc42 activity is absolutely required for IL-1-induced actin polymerization and remodeling processes (Puls et al., 1999). Exposure of HeLa cells to IL-1 results in the activation of RhoA, which in turn leads to the formation of stress fibers. Moreover, IL-1 RI, in a biochemical affinity purification protocol, was found to be complexed with RhoA and Rac1 (Singh et al., 1999). It will be interesting to see, whether the newly identified MRX-related IL1RAP protein also will involve Rho GTPases as part of its general signaling potential in the brain.

Tetraspanins have been observed in protein complexes with adhesion molecules such as α - and β -integrins. The TM4SF proteins CD151, CD81 and CD63 have been found to be associated with $\alpha\beta$ 1-integrins in neurites and growth cones of human NT2N cells. When treated with antibodies against CD151 and CD81, neurite outgrowth in NT2N cells grown on $\alpha\beta$ 1 integrin-specific extracellular matrix molecules (ECM) was greatly impaired. Under these conditions, neurite number, length and extension rate were all affected (Stipp and Hemler, 2000). As there appears to be a general requirement for Rho GTPase activity in axon and neurite outgrowth and retraction, as well as a functional connection between integrin and Rho signaling it would not be surprising to find that TM4SF/integrin complexes function in concert with the Rho GTPases (Jalink et al., 1994; Luo et al., 1994). Intriguingly, in a recent study, a TM4SF-family member was netted as a binding partner of oligodendrocyte-specific protein (OSP/Claudin-11), a member of the expanding Claudin family, which is responsible for establishing tight junction-equivalent structures in myelin-forming oligodendrocytes of the CNS. For this reason, the respective TM4SF protein was named OAS for OSP/Claudin-11 associated protein 1 (Tiwari-Woodruff et al., 2001). Furthermore, β 1-integrin subunits were recovered as another constituent of OAS/OSP complexes (Tiwari-Woodruff et al., 2001). Loss of OSP/Claudin-11 in gene-targeted mice results in the absence of tight junction-like structures in CNS myelin sheaths, which induces neurological abnormalities (Gow et al., 1999). Interference experiments using OAP-1- and OSP/Claudin-11-specific antibodies in primary oligodendrocytes effectively impaired migration, whereas, overexpression of OAP-1 or OSP/Claudin-11 caused an oligodendrocytic cell line to overproliferate in culture (Tiwari-Woodruff et al., 2001). In light of the well-established role of Rho proteins in determining the morphology and function of tight junctions in epithelial cells (Jou et al., 1998), these data may hint at the possibility of oligodendrocytic defects being important in the genesis of MRX. It remains to be seen which specific cell type

TM4SF2 will be functional in. Its wide spread expression pattern so far does not exclude any of the mentioned possibilities, all of which may well be molecularly and physiologically linked to Rho GTPase signaling.

Finally, a potentially intriguing role for Rac1 signaling in the development of the fragile X mental retardation syndrome has been suggested (Schenck et al., 2001). FMRP (fragile X-linked mental retardation protein) is the product of the FMR1 gene, mutations in which manifest themselves in the Fragile X mental retardation syndrome (FraX). FraX can be distinguished from non-syndromic X-linked mental retardation in that it is also associated with other phenotypes such as macroorchidism, large ears, prominent jaws and a high-pitched jocular speech in affected individuals (Hagerman, 1996; Imbert et al., 1998). In FraX, the FMR1 gene is transcriptionally silenced due to a hypermethylated CGG repeat expansion in the sequence encoding the 5'-untranslated region (Imbert et al., 1998). FMRP harbors nuclear export and import signals, as well as multiple mRNA binding motifs (Ashley et al., 1993; Siomi et al., 1993; Eberhart et al., 1996; Sittler et al., 1996; Tamanini et al., 1999). Thus it may serve to shuttle specific mRNAs between the nucleus and the cytoplasm. The finding that the concentration of FMRP is high in neurons and particularly within dendritic processes suggests that it may direct its cargo mRNAs to a specialized cellular compartment for more efficient translation (Devys et al., 1993; Weiler et al., 1997; Jin and Warren, 2000). More recently, FMRP has been shown to associate with CYFIP1 (cytoplasmic FMRP interacting protein 1), a component of the synaptosome (Schenck et al., 2001). In fact, CYFIP1 was a known protein that earlier had been described as a Rac1 interacting protein, termed p140Sra-1, which was observed to be associated with Rac-induced cortical actin filaments and to cosediment with F-actin (Kobayashi et al., 1998). It is tempting to speculate that activated Rac1, together with CYFIP1, tethers FMRP to developing spines to spatially control the translation of spine-relevant cargo mRNAs. Rac1 activation, in this scenario, may result from activation of receptors for neurotransmitters in spine synapses. Mutational down-regulation of FMRP may interfere with such translational processes, which ultimately produces FraX-associated phenotypes.

As illustrated above, a number of MRX genes are either evident components of Rho GTPase signaling cascades or may be indirectly linked to the activities of Rho GTPases. Since each of the cloned MRX genes accounts for only 0.5–1.0% of the total MRX cases (Chelly, 2000), one can predict a considerable number of additional MRX genes to surface in the future. It is very likely that among these MRX genes additional components of Rho GTPase signaling pathways will be identified. Tying in MRX-defects on their physiological level with the molecular details of Rho GTPase signaling will be a major challenge. Experiments addressing whether the Rho GTPase-related MRX genes and their products found thus far may cooperate in a common path-

way is an interesting starting point. Also the development of animal models carrying targeted disruptions in MRX genes and transgenes that can be conditionally expressed will be beneficial to these aims. Defining these Rho GTPase-controlled pathways and integrating them into a general neuronal signaling network will contribute to understanding the molecular details of cognitive processes.

3.2. Motor neuron degeneration and Down syndrome

3.2.1. *Alsin/ALS2 in amyotrophic lateral sclerosis (ALS)*

A major breakthrough in the search of genetic mutations causing familial amyotrophic lateral sclerosis (ALS) has been the recent cloning of a locus on chromosome 2q33 encoding a putative novel RhoGEF by two independent groups (Hadano et al., 2001; Yang et al., 2001; Fig. 2). Accordingly, one of the groups coined the term *alsin* (ALSin) for the respective protein product.

ALS is a progressive paralytic disorder that affects motor neurons in different regions of the nervous system. Due to genetic lesions, upper motor neurons (UMN) in the motor cortex as well as lower motor neurons (LMN) in the brainstem and spinal cord are rendered dysfunctional and degenerate. This causes the affected individuals to die most frequently from respiratory failure, often within 3 years after onset of the disease. Whereas 90% of the ALS cases are sporadic, 10% are of familial origin. The familial cases harbor genetically transmitted, usually autosomal dominant mutations. In a small percentage of familial ALS (ALS2) the first clinical manifestations are detectable before the age of 25, with the disease developing at a slower pace than in the rest of the familial ALS patients.

The first, and for a long time only, mutated ALS-gene identified was the one coding for Cu/Zn superoxide dismutase (SOD1) (Rosen, 1993). SOD1's catalytic activity reduces the amount of free radicals in virtually all cell types and ALS-specific mutations are thought to render SOD1 hyperactive rather than inactive, suggesting the necessity for a delicate balance in the number of free radicals for motor neuronal processes. According to estimations based on multiple mapping efforts, the SOD1 locus only accounts for about 20% of familial ALS cases arising from autosomal dominant mutations. Aberrations in other genes were eagerly awaited.

The *alsin* gene was the second locus to be involved in the neurodegenerative processes underlying ALS, in particular the juvenile form of ALS, ALS2. The discovery of *alsin* as a putative Rho GTPase activator could open new vistas on the ill-defined biochemical pathways that if impaired can cause ALS. The microdeletions found in the *alsin* gene disrupt its coding unit, and thus generate loss-of-function alleles (Hadano et al., 2001; Yang et al., 2001). The *alsin* protein, apart from a typical tandem DH/PH domain, contains two types of motifs that are also present in other GTP/GDP exchangers for Ras GTPases. Aligned in its N-terminus are three copies of the RCC1-like domain (RLD), which

was originally described for the Ran GTPase-specific exchange factor RCC1. RCC1 associates with chromatin and regulates the nuclear-cytoplasmic shuttling of Ran. At its C-terminus, *alsin* harbors a VPS9 domain that is shared by exchange factors mediating vacuolar protein sorting in yeast in the case of VPS9 itself, or endocytic trafficking in the case of VSP9 domain-containing eukaryotic GEFs. In addition, *alsin* contains an array of MORN (membrane occupation and recognition nexus) motifs that very recently have been implicated in the membrane association of junctophilins (Takeshima et al., 2000). Junctophilins comprise a novel protein family that is present at junctional complexes established between the plasma membrane and extensions of the endoplasmic/sarcoplasmic reticulum, which are features of excitable cell types and are thought to mediate communication between the plasma membrane and intracellular ion channels.

All of the listed consensus motifs may contribute to *alsin*'s specific subcellular location and function, but the specific role of *alsin* in motor neurons is illusive at this point. Pivotal issues concerning the Rho GTPase-specificity and the sub-neuronal localization of *alsin* still need to be resolved to solidify the basis for further speculation. However, in light of the disease phenotype that is produced by *alsin*-specific mutations, namely the degeneration of motor neuronal circuits, it is intriguing to speculate on a more general role of Rho GTPase-dependent signaling for the maintenance of neuronal pathways. The disruption of such pathways might provoke neurodegenerative processes.

3.2.2. *Intersectin as a potential component in Down syndrome*

The intersectin-encoding gene on chromosome 21q22 has been speculated to contribute to the malignant aspects of Down syndrome (Guipponi et al., 1998). Intersectin, which represents yet another member of the RhoGEF family has been implicated into endocytic events and mitogenic processes and might even link the two (Fig. 2). Mechanistically, intersectin stimulates Cdc42 and thus regulates N-WASP/Arp2/3-dependent actin polymerization, possibly at sites of active endocytosis (Hussain et al., 2001; O'Bryan et al., 2001). The classification of intersectin as a genuine disease molecule may be premature at this point, but the localization of the encoding gene on chromosome 21 and elevated expression levels of intersectin-specific mRNA in patients suffering from Down syndrome make it an interesting molecule in the context of trisomy 21 research (Guipponi et al., 1998; Pucharcos et al., 2000, 2001).

4. Other potentially Rho GTPase-related disabilities

4.1. *FGD1 (faciogenital dysplasia)*

By conventional means of forward genetics, the FGD gene has been cloned and revealed to be the mutated

locus causing faciogenital dysplasia, also known as Aarskog–Scott syndrome (Pasteris et al., 1994). The discovery of additional mutant FGD-alleles since then has confirmed the role of the gene in the development of the disease (Orrico et al., 2000; Schwartz et al., 2000). Faciogenital dysplasia is an X-linked developmental disorder and individuals are of disproportionately short stature and suffer from facial, skeletal and urogenital abnormalities. The FGD gene product, FGD1, was predicted to function as a Rho-specific GEF (Fig. 2), and the disease-causing mutation at first identified by Pasteris et al. was predicted to insert a premature stop codon into the region encoding the protein's DH-function. FGD1, apart from the DH-motif also contains the juxtaposed PH-domain typical of Rho GEFs, as well as a number of SH3-binding regions and an additional C-terminal PH-domain (Pasteris et al., 1994). Subsequently, Zheng and colleagues have demonstrated that FGD1 exerts its activity specifically on Cdc42 (Zheng et al., 1996). Further dissection of the FGD1 molecule determined that the DH-domain alone can induce G1 progression when introduced into fibroblasts, but that both the DH and the contingent PH sequence are needed to induce a Cdc42-specific response of the actin cytoskeleton, namely the formation of filopodia and microspikes (Nagata et al., 1998). This result suggests that proper recruitment of FGD1 to actin-relevant subcellular sites depends on proper PH-function. Interestingly, mutational analysis of another FGD pedigree by Orrico et al. (2000) has revealed a R610Q amino acid substitution. Arg-610 resides in the PH-domain of the DH/PH-motif and appears to be a conserved residue in PH-domains that are involved in inositol-phosphate (InsP)-binding (Ferguson et al., 1995; Salim et al., 1996). Together, these results suggest that FGD1 is targeted by specific InsP-species to distinct membranes where it exerts its GEF activity towards Cdc42. To date, no upstream activating mechanism for FGD1-specific signaling has been described but the strong similarity between the PH-domain harboring Arg-610 and the one of the β -adrenergic receptor kinase (ARK) suggests that PtdIns-4,5-P₂ and/or PtdIns-3,4,5-P₃ might mediate the recruitment of FGD1 to membrane domains. ARK has been shown to associate with these InsP species (Rameh et al., 1997).

4.2. WASP (*Wiskott–Aldrich syndrome*)

Two other genetic disorders in which mutations affect Cdc42 signaling are the X-linked Wiskott–Aldrich syndrome (WAS) and the related allelic X-linked thrombocytopenia (XLT). As with FGD and the MRX genes, the gene causing WAS and XLT is located on the X-chromosome, rendering recessive alleles (approximately 50 different mutations have been detected so far in the WAS gene) to phenotypic expression (Derry et al., 1995; Kolluri et al., 1995; Kwan et al., 1995; Villa et al., 1995; Wengler et al., 1995; Zhu et al., 1995). Defects are restricted to hematopoietic lineages and include microthrombocytopenia and

recurrent infections because of dysregulated T- and B-cell functions. The observations that neutrophils are enabled in their proper chemotactic response and that B cells do not show their typical response to polysaccharides, together with the severe cytoskeletal defects observed in T cells and platelets, suggested an actin-related role for the WAS gene product. Derry et al. succeeded in cloning the gene responsible for WAS and through rigorous screening assembled the first set of WAS-specific mutations including nonsense, missense and frame shift mutations (Derry et al., 1994). Subsequently, in biochemical overlay assays designed to find novel Cdc42-effectors, Symons et al. isolated WASP (for WAS protein) from human neutrophils (Symons et al., 1996). WASP appeared to bind exclusively to GTP-loaded Cdc42 and not to Rac1 or RhoA, and was traced in high concentrations to polymeric actin structures. Through the work of several groups a picture emerged, in which Cdc42, in concert with WASP, stimulates the Arp2/3 complex which in turn enables actin filaments to nucleate from germination centers (for review see Welch, 1999; Fig. 2). This activity is required for morphological cell shape changes and cell migration, processes that are impaired in WASP-deficient hematopoietic cells. Accordingly, WASP transcripts were found in compartments of the immune system, such as the spleen, thymus and lymphocytes (Derry et al., 1994). Notably, a homologue of WASP, namely N-WASP is expressed ubiquitously, with particularly high expression levels in the nervous system, and fulfills WASP functions in cells other than those of hematopoietic origin (Miki et al., 1996). In subsequent experiments, N-WASP was shown to be an indispensable element in the Cdc42-dependent pathway leading to the formation of filopodia in fibroblasts (Miki et al., 1998). WASP, moreover, appears to be subject to precise regulatory mechanisms to guarantee its proper function. This has become even more apparent when Devriendt et al. recently reported that another hematopoietic deficiency, namely X-linked severe congenital neutropenia (XLN), is caused by a WAS mutation that generates a constitutively activated WASP protein (Devriendt et al., 2001). A single amino acid substitution at position 270 from Leu to Pro in the autoinhibitory domain of WASP was detected, and based on structural data by Kim and co-workers (Kim et al., 2000) is shown to map to the Rho GTPase binding domain (Kim et al., 2000; Devriendt et al., 2001). These findings underscore the *in vivo* importance of the Cdc42/WASP interaction once more.

4.3. *Diaphanous* (*non-syndromic deafness*)

Another example of a gene encoding a disease-related Rho GTPase effector is *Diaphanous* 1 (also known as DFNA1). An effort to identify the autosomal dominant mutation responsible for nonsyndromic deafness led to the cloning of a gene on chromosome 5q31 that turned out to be homologous to the *Drosophila diaphanous* gene (Lynch et al., 1997). The first clue hinting at a possible function for

diaphanous came from another source. Watanabe and co-workers, in a quest for RhoA-specific binding partners, identified p140mDia/mDia1, the mouse orthologue of the diaphanous protein (Fig. 2). They further showed that mDia1 was not only tethered to activated Rho by its N-terminus, but that it also serves as a ligand for Profilin via its formin homology (FH1) domain (Watanabe et al., 1997). This interaction is believed to stimulate actin remodeling at particular locations in the cell in response to Rho activation. Indeed, in a follow-up study, mDia1 turned out to work in concert with ROCK to induce stress fibers in transfected fibroblasts (Watanabe et al., 1999). Interestingly, mDia1 caused the alignment of actin together with microtubule filaments in a coordinated bipolar fashion when transfected into HeLa cells. This activity was ascribed to the C-terminal FH2 domain of the molecule and suggests mDia1/diaphanous as an actin/microtubule coordinating factor downstream of Rho (Ishizaki et al., 2001). Similarly, Palazzo et al. found mDia1 to colocalize with microtubules and to trigger the formation and orientation of stable microtubules in serum-starved fibroblasts (Palazzo et al., 2001). Deafness, arising from a mutation in the diaphanous gene is associated with a sensorineural cochleosaccular dysplasia of the membranous structures of the inner ear. It has been speculated that diaphanous may act in a pathway linking integrins to the actin cytoskeleton. Mutations in $\alpha 8 \beta 1$ integrins, diaphanous and myosin VIIa (as well as other myosins) all cause deafness, indicating that the wild-type proteins could organize and/or maintain cytoskeletal structures in hair cells of the inner ear (Lynch et al., 1997; Richardson et al., 1999; Littlewood Evans and Muller, 2000; for a review about deafness-related genes see Muller and Littlewood-Evans, 2001).

4.4. A potential role for Rho GTPases in Tangier disease

In the last 'case study' presented in this review, Rho GTPase function has been correlated with Tangier disease. The basis of this disease is an impaired cholesterol efflux (CE) mechanism, resulting in abnormally low high density lipoprotein (HDL) plasma levels and an accumulation of cellular cholesteryl esters. Thus, CE is an essential process that purges excess cholesterol from cells and provides an important protection against arteriosclerosis. Examination of fibroblasts and macrophages from Tangier patients by cDNA subtraction techniques revealed a substantial down-regulation of Cdc42 levels in affected cells. Accordingly, Hirano et al. found that introduction of a dominant negative form of Cdc42 into epithelial MDCK cells could decrease CE efficiency and that dominant active Cdc42 could increase it (Hirano et al., 2000). In addition, RhoA, RhoB, RhoG and Rac1 levels appeared to be elevated in fibroblasts derived from Tangier patients (Utech et al., 2001). Whether Rho GTPases play a direct role in Tangier disease, and how a particular Rho GTPase affects aspects of CE, remains

elusive, but future research into this subject may well shed light onto yet another aspect of Rho GTPase signaling.

4.5. Rho GTPases and bacterial infections

Through work over the last few years, it has become clear that the manipulation of Rho GTPases is a step in a number of disease-causing bacterial infections. Rho GTPases are manipulated by bacterial toxins from quite different origins and of different make and function. For more detail, we refer to recent reviews that treat this subject with much greater depth (Lerm et al., 2000; Stebbins and Galan, 2001). To summarize the recent research, Rho GTPases are targeted by basically three classes of toxins that display fundamentally different activities. The first class contains the Rho GTPase-inactivating C3 exotoxins and LCC (large clostridial toxin), toxins that act as ADP-ribosyltransferases and glucosyltransferases, respectively. The covalent attachment of ADP-ribosyl-moieties by the C3-transferases of *Clostridium botulinum*, *Staphylococcus aureus* and other infectious bacteria blocks the ability of RhoA, B and C to interact with Rho-specific GEFs and thus prevents activation of these GTPases. Glucosylation of Rho at Thr-37 and Rac and Cdc42 at Thr-35 by the large LCC toxins (larger than 250 kDa) which is produced by *Clostridium* spp., interferes with nucleotide binding and coordination of the nucleotide-linked Mg^{2+} -ion. This in turn inhibits the association of effector molecules with the same region of the GTPases and abrogates the stimulation of downstream signaling pathways. The second class of toxins contains the Rho GTPase-deamidating CNF (cytotoxic necrotizing factor) agents from *Escherichia coli* and DNT (dermonecrotic toxin) toxins from *Bordetella* spp. CNF toxins deamidate Gln-63 in Rho and Gln-61 in Rac and Cdc42. Since these residues are essential for GTP-hydrolysis and therefore inactivation of the Rho GTPases, this renders them constitutively active. This results in an overactivation of particular Rho-dependent signaling pathways. DNT toxins are transglutaminases that constitutively activate Rho by adding an amine to Gln-63.

A particularly interesting class of toxins is the one of 'injected' toxins, that due to a specialized 'type III' secretion system are directly introduced from *Yersinia* and *Salmonella* bacteria into their host cells. 'Injected' toxins mimic cellular GEFs and GAPs to a degree that allows the invading bacterium to first activate Rho GTPases, such as Rac1 and Cdc42 in the case of SopE from *Salmonella* spp. This ensues remodeling of the actin cytoskeleton to promote bacterial entry. Post internalization, another class of 'injected' toxins, exemplified by SptP from *Salmonella* spp. and YopE from *Yersinia* spp. is produced, this time to reversibly inactivate Rho GTPases by mimicking host GAPs. Thus, deregulation of cytoskeletal structures is reversed and the host cell saved for the future benefit of the invaders.

Recent structural analysis has unraveled the basis for some of these mechanisms. It appears that through conver-

gent evolution *Salmonella* spp. and *Yersinia* spp. have developed proteins that, although they do not share the tertiary structures of the enzymes they mimic, nevertheless display contact surfaces and identical residues at critical positions that allow for catalysis (Lerm et al., 2000; Stebbins and Galan, 2001). The corruption of Rho GTPase activities in the interest of pathogenic infection can be regarded as a redundant theme and the different strategies that have been evolutionarily employed by bacteria to this end are diverse and fascinating. The observation that overactivation as well as underactivation of Rho GTPases occurs in the course of bacterial attacks suggests that Rho GTPase signaling is being exploited in some cases and disrupted in others. In this sense, the strategies used by *Salmonella* spp. seem even more sophisticated. The C3-transferase from *C. botulinum* already has been used successfully as an experimental tool to inhibit Rho-function in diverse systems (see e.g. Cleverley et al., 2000). It is conceivable, that other Rho GTPase-specific toxins will be introduced as more generally applicable reagents into research that pertains to clinically relevant questions.

5. Conclusions and future perspectives

Given the complexity of Rho GTPase signaling and the multiple cellular and developmental aspects involving and requiring the function of Rho GTPases, there is a very strong possibility that many more disease-causing mutations in genes encoding Rho-related signaling molecules will be uncovered in the future. The available annotated genome sequences suggest a vast number of genes for Rho GTPase-specific regulators, but many of them remain biologically unexplored to date. Emerging areas of interest in which the contribution of Rho GTPases is currently being investigated include mechanisms of viral infection and various organ- and tissue-specific diseases, such as ventricular myocyte hypertrophy underlying some dysfunctions of the heart. However, due to lack of compelling evidence that would definitively prove the corruption of a Rho GTPase-related signaling molecule, we chose not to elaborate on these processes.

The following points illustrate some of the possibilities that will influence future research to provide us with a better concept of how Rho GTPases function in numerous disease aspects. (1) The findings that Wnt- and Ras-signaling may feed into Rho-signaling to manifest some Rho GTPase-dependent phenotypes emphasize the interdependency and collaboration of different signaling pathways during the progression of a given disease. Other disease-relevant pathways will be linked to Rho-signaling in the years to come. Adhesion-triggered signaling in various forms is a good candidate for this. (2) Rho-specific activities in the establishment of cellular polarity and cell-cell adhesive properties, as well as their impact on vesicle trafficking, may be of importance for the development of different diseases. Also

for this reason, studies in these areas merit further attention. (3) Along the same lines, the MRX genes encoding components and potential components of Rho GTPase signaling pathways that have been identified thus far will have to be functionally connected to an operational network. The establishment of mouse model systems will help in understanding the contribution of these genes and their interactions on a physiological level.

It is evident that the biological activities of the disease genes elaborated upon in this review will exceed the scope of the context in which they were initially identified. Research addressing these aspects may ultimately also help design strategies for therapeutic intervention and diagnostic purposes. Certainly, the interplay and collaboration between disease research on the one hand, and biochemical, as well as cell biological, research on the other will further help advance these subjects and activate our individual levels of excitement.

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